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Haojian Zhang

*University of Massachusetts Medical School*

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**THE MOLECULAR MECHANISMS FOR MAINTENANCE OF  
CANCER STEM CELLS IN CHRONIC MYELOID LEUKEMIA**

**A Dissertation Presented**

**By**

**HAOJIAN ZHANG**

**Submitted to the Faculty of the  
University of Massachusetts Graduate School of Biomedical Sciences, Worcester  
in partial fulfillment of the requirements for the degree of**

**DOCTOR OF PHILOSOPHY**

**MAY 23, 2012**

**Cancer Biology Program**

**THE MOLECULAR MECHANISMS FOR MAINTENANCE OF  
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## Abstract

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder associated with the Philadelphia chromosome (Ph) that arises from a reciprocal translocation between chromosomes 9 and 22, thereby resulting in the formation of the chimeric *BCR-ABL* oncogene encoding a constitutively activated tyrosine kinase. BCR-ABL tyrosine kinase inhibitors (TKIs) induce a complete hematologic and cytogenetic response in the majority of chronic phase CML patients. However, TKIs cannot efficiently eradicate leukemia stem cells (LSCs) because of the insensitivity of LSCs to TKIs. Therefore, developing new strategies to target LSCs is necessary and critical for curing CML, and success of this approach depends on further understanding the molecular mechanisms by which LSCs survive and are maintained.

In Chapter I, I briefly introduce CML disease, BCR-ABL oncoprotein, and TKIs. I also describe the identification and features of LSCs. Several key pathways in LSCs including Wnt/ $\beta$ -catenin, hedgehog, FoxO, Bcl6 and HIF1, are discussed. I also propose our strategy to identify unique molecular pathways that are important for LSCs but not their normal stem cell counterparts.

In Chapter II, I describe our finding about the function of the positive regulator, HIF1 $\alpha$ , in CML development and LSC survival. I show that loss of *HIF1 $\alpha$*  impairs the maintenance of CML through impairing cell cycle progression and inducing apoptosis of LSCs, and I also report that p16<sup>Ink4a</sup> and p19<sup>Arf</sup> mediate the effect of HIF1 $\alpha$  on LSCs, as knockdown of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> rescues the defective colony-forming ability of *HIF1 $\alpha$* <sup>-/-</sup> LSCs.

As detailed in Chapter III and IV, through comparing the global gene expression profiles of LSCs and HSCs, I find two novel regulators, *Blk* and *Scd1*, which act as tumor suppressors in CML development. In Chapter III, I show that *Blk* is markedly down-regulated by BCR-ABL in LSCs, and that c-Myc and Pax5 mediate this down-regulation. Deletion of *Blk* accelerates CML development; conversely, *Blk* overexpression significantly delays the development of CML and impairs the function of LSCs. I also demonstrate that p27, as a downstream effector, is involved in the function of *Blk* in LSCs. *Blk* also functions as a tumor suppressor in human CML stem cells, and inhibits the colony-forming ability of human CML cells. In Chapter IV, I investigate the function of another negative regulator, *Scd1*, in CML LSCs, and find that expression of *Scd1* is down-regulated in mouse LSCs and human CML cells. We report that *Scd1* acts as a tumor suppressor in CML, as loss of *Scd1* causes acceleration of CML development and overexpression of *Scd1* delays CML development. Using a colony-forming assay, I demonstrate that *Scd1* impairs the maintenance of LSCs due to the change of expression of Pten, p53 and Bcl2. Importantly, I find that both *Blk* and *Scd1* do not affect normal hematopoietic stem cells (HSCs) or hematopoiesis.

Taken together, our findings demonstrate that HIF1 $\alpha$  is required for the maintenance of CML LSCs, and conversely that *Blk* and *Scd1* suppress the function of LSCs, suggesting that combining TKI treatment with specific targeting of LSCs will be necessary for curing CML.

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Figure 1. The combination of inhibiting BCR-ABL by TKIs and targeting key regulators of LSCs in CML treatment.

### List of Abbreviations

CML	Chronic myeloid leukemia
Ph	Philadelphia chromosome
AML	Acute myeloid leukemia
GRB2	Growth factor receptor bound protein 2
5-FU	5-fluorouracil
IL-3	Interleukin-3
IL-6	Interleukin-6
SCF	Stem cell factor
MT	Metallothionein
MMTV-LTR	Mammary tumor virus long terminal repeat
tTA	Tetracycline transactivator
SCL	Stem cell leukemia
MACS	Magnetic-activated cell <i>sorting</i>
FACS	Fluorescence-activated cell sorting
TKI	Tyrosine kinase inhibitor
SFK	Src family kinase
LSC	Leukemia stem cell
CSC	Cancer stem cell
CFSE	Carboxy-fluorescein diacetate succinimidyl diester
CFC	Colony forming assay
LTC-IC	Long-term bone marrow culture-initiating cells

CMP	Common myeloid progenitor
GMP	Granulocyte-macrophage progenitors
CLP	Common lymphoid progenitor
BMT	Bone marrow transplantation
PI3K	Phosphoinositide 3-kinases
STAT	Signal transducers and activator of transcription
Blk	B lymphoid kinase
Alox5	Arachidonate 5-lipoxygenase
Scd1	Stearoyl-Coenzyme A desaturase 1
FOXO	Forkhead O
HIF1	Hypoxic inducible factor 1
GSK3 $\beta$	Glycogen synthase kinase 3 $\beta$
CK1	Casein kinase 1
APC	Adenomatous polyposis coli
Hh	Hedgehog
Shh	Sonic hedgehog
Ihh	Indian hedgehog
Dhh	Desert hedgehog
BCL6	B cell lymphoma 6
LXR	Liver X receptor
SREBP-1c	Sterol response element binding protein 1c
PPAR	Peroxisome proliferator activated receptor

B-ALL	B-cell acute lymphoblastic leukemia
MUFA	Monounsaturated fatty acid
SFA	Saturated fatty acid
IR	Insulin receptor
IRS	Insulin receptor substrate
Pten	Phosphatase and tensin homolog
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
Bcl-2	B-cell lymphoma 2
Wnt	wingless-related MMTV integration site
bHLH	Basic helix-loop-helix
PHD	Proline hydroxylase
pVHL	Von Hippel-Lindau protein
VEGF	Vascular endothelial growth factor
Glut1	Glucose transporter type 1
TGF $\alpha$	Transforming growth factor alpha
PGK1	Phosphoglycerate kinase 1
GSEA	Gene set enrichment assay
FISH	Fluorescence in situ hybridization
Flt3	Fms-related tyrosine kinase 3
IGF-2	Insulin like growth factor 2
TPO	Thrombopoietin
FGF	Fibroblast growth factor

Pax5	Paired box protein 5
EBF1	Early B-cell factor 1
Skp2	S-phase kinase-associated protein 2



## Preface

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**Haojian Zhang**, Huawei Li, Ngoc Ho, Dongguang Li, Shaoguang Li. Scd1 plays a tumor suppressive role in survival of leukemia stem cells and the development of chronic myeloid leukemia. *Mol. Cell. Biol.* 2012 32: 1776-1787.

Con Sullivan, Yaoyu Chen, Yi Shan, Yiguo Hu, Cong Peng, **Haojian Zhang**, Linghong Kong, Shaoguang Li. Functional Ramifications for the Loss of P-Selectin Expression on Hematopoietic and Leukemic Stem Cells. *Plos One* 2011 6 (10):e26246

Peng C, Chen Y, Yang Z, **Zhang H**, Osterby L, Rosmarin AG, Li S. PTEN is a tumor suppressor in CML stem cells and BCR-ABL-induced leukemias in mice. *Blood* 2010 115(3):626-35

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## CHAPTER I

### INTRODUCTION

#### 1. Chronic myeloid leukemia and the Philadelphia chromosome

Chronic myeloid leukemia (CML) is a myeloproliferative disease that originates from an abnormal hematopoietic stem cell (HSC) harboring the Philadelphia (Ph) chromosome (Figure 1A)<sup>1-3</sup>. CML is a rare disease with an incidence of 1 or 2 cases per 100,000 people every year in United States<sup>4</sup>. It occurs most commonly in the middle-aged and elderly, and accounts for 15-20% of all cases of adult leukemia<sup>4</sup>. In general, CML has three distinct clinical stages: the initial indolent chronic phase, the accelerated phase and the blast crisis<sup>1,4</sup>. The chronic phase is characterized by the accumulation of myeloid precursors in the bone marrow and mature granulocytes in peripheral blood<sup>4</sup>. The accelerated phase is characterized by an increase in disease burden and in the frequency of progenitor cells. Blast crisis is characterized by arrested hematopoietic differentiation and the accumulation of immature blast cells resembling acute leukemia<sup>4</sup>. About 70% of CML patients develop acute myeloblastic leukemia (AML), and approximately 20-30% develop acute lymphoblastic leukemia (ALL)<sup>1</sup>.

The discovery of the Philadelphia chromosome by Nowell and Hungerford in 1960 through analyzing leukemic cells from seven chronic phase CML patients<sup>5</sup>, provided a major clue to the pathogenesis of CML. This chromosomal abnormality is generated by a reciprocal translocation between chromosome 9 and 22 [t (9; 22)(q34; q11)], which results in the formation of the chimeric BCR-ABL protein that functions as

a constitutively active tyrosine kinase<sup>1,6</sup>. This aberrant BCR-ABL oncoprotein constitutes the molecular basis of CML.

## 2. BCR-ABL oncoprotein

The product of the Philadelphia chromosome was identified by diverse experimental approaches<sup>7-10</sup>, showing that the *ABL* proto-oncogene on chromosome 9 translocates into the 5' sequence of the breakpoint cluster region (*BCR*) gene on chromosome 22, thereby generating the fusion oncogene *BCR-ABL*, which encodes the BCR-ABL oncoprotein<sup>7,9</sup>. Furthermore, advances in chromosome mapping and molecular biology enabled the discovery of additional forms of BCR-ABL, including p185<sup>BCR-ABL</sup>, p210<sup>BCR-ABL</sup>, and p230<sup>BCR-ABL</sup>, which are generated due to different breakpoints in the *BCR* gene (Figure 1B)<sup>8</sup>. The first breakpoint is the minor breakpoint cluster (m-bcr), which results in a smaller 7.0 kb mRNA that generates p185<sup>BCR-ABL</sup>; the major breakpoint cluster (M-bcr) results in an 8.5 kb mRNA encoding p210<sup>BCR-ABL</sup>; another minor breakpoint is  $\mu$ -bcr which exists at the end of the *BCR* gene and generates an 9.0 kb mRNA coding for p230<sup>BCR-ABL</sup><sup>1,8</sup>. It is known that these distinct forms are associated with relatively specific human leukemias: p185<sup>BCR-ABL</sup> is mainly associated with 66% of adult B-ALL<sup>1</sup>; the majority of CML cases are caused by p210<sup>BCR-ABL</sup>; p230<sup>BCR-ABL</sup> is mainly identified in neutrophilic CML as well as some CML cases<sup>1</sup>.

ABL kinase belongs to the non-receptor tyrosine kinase family and has three highly conserved domains, including an SRC-homology 3 (SH3), an SH2 and a tyrosine kinase domain<sup>6</sup>. In the carboxy-terminus, ABL has an actin binding domain (ABD), a DNA binding domain (DBD), three nuclear localization signals (NLSs), and four proline-

rich SH3 binding sites (PPs)<sup>6</sup>. These domains assemble ABL into a dynamic protein that can switch between inactive and active conformations<sup>6</sup>. The N-terminal myristoyl group or hydrophobic residues in the cap domain of ABL bind to the deep hydrophobic pocket in the kinase domain, which contributes to the maintenance of an inactive conformation of ABL<sup>11</sup>. Previous studies demonstrated that loss of myristoylation in ABL dramatically increased the tyrosine kinase activity of ABL by promoting the switch of an inactive conformation to an active state<sup>11,12</sup>. These findings suggested that myristoylation of ABL inhibits its kinase activity. The latching of the N terminus of ABL to its kinase domain places the SH3 and SH2 domains on the backside of the small and large lobes of the tyrosine kinase domain respectively, resulting in the clamping of these structures and stabilization of the inactive conformation of ABL<sup>1</sup>. This was confirmed by the findings that mutations in the SH3/SH2 regions abolished the clamping and resulted in the activation of ABL<sup>13</sup>. Additionally, the activation loop of the kinase domain is sequestered in this clamping structure, thus preventing the phosphorylation of tyrosine 412 that is required for full activation of the kinase<sup>6</sup>. Therefore, the crystal structure of ABL implies that disruption of these inhibitory processes might be the mechanism by which chimeric oncoprotein BCR-ABL attains constitutive kinase activity<sup>6</sup>.

As a component of the fusion protein, BCR is a serine/threonine kinase protein<sup>6</sup>. BCR contains several domains, including a coiled-coil (CC) oligomerization domain, A and B boxes, a serine/threonine kinase domain, a Dbp/CDC24 guanine-nucleotide exchange factor homology domain, a pleckstrin homology domain, a putative calcium-dependent lipid binding site (CaLB) and a RAC guanosine triphosphatase-activating

protein (RAC-GAP) domain<sup>6</sup>. These domains have been shown to activate tyrosine kinase activity and increase the transforming capability of BCR-ABL<sup>14,15</sup>. First, the CC oligomerization domain of BCR contributes to the activation of BCR-ABL<sup>14</sup>. McWhirter et al. demonstrated that disruption of the CC oligomerization domain of BCR by insertional mutagenesis inactivates the kinase activity and the transformation ability of BCR-ABL<sup>14</sup>. This finding suggested that the CC oligomerization domain of BCR correlates with the activation of the tyrosine kinase of BCR-ABL. Mice transplantation experiments also showed that mutant BCR-ABL without the BCR CC domain failed to induce myeloid leukemia, suggesting a critical role for this domain<sup>15</sup>. Second, the A and B boxes are required for BCR-ABL activation and cellular transformation<sup>16,17</sup>, as perturbations through deletions or substitutions can disrupt their binding to the SH2 domain of ABL, which is required for maintaining BCR-ABL in an active conformation<sup>16,17</sup>. Additionally, previous data also showed that phosphoserine/phosphothreonine but not phosphotyrosine residues on BCR are required for interaction with the ABL SH2 domain<sup>17</sup>. Therefore, BCR encoded domains activate the tyrosine kinase and the transforming potential of BCR-ABL through specifically interfering with negative regulation of the ABL-encoded tyrosine kinase. Finally, the tyrosine at residue Tyr177 (Y177) located in the first exon of BCR is critical for BCR-ABL binding to growth factor receptor bound protein 2 (GRB2) and induction of the downstream RAS signaling pathway<sup>15</sup>. Mutant Y177 blocks this binding function and fails to induce myeloid leukemia<sup>15</sup>.

### 3. CML mouse models

A good animal model of CML will help us study the pathogenesis of CML and characterize leukemic and normal stem cells, and contribute to the evaluation of experimental therapeutic strategies for this disease. Several CML mouse models have been established. The most widely used model is the retroviraltransduction/transplantation model (Figure 2A)<sup>18-22</sup>. In this model, murine bone marrow cells are infected with a retrovirus encoding p210<sup>BCR-ABL</sup> and transplanted into irradiated syngeneic recipients<sup>18,20</sup>. Over time, the recipients develop a CML-like myeloproliferative syndrome. Originally, this early system was limited because of difficulties in generating high-titer BCR-ABL retroviruses, which subsequently caused a low efficiency of generating diseased animals<sup>18,20</sup>. Additionally, serial transfer of the myeloproliferative disease also occurred at a low frequency<sup>18,20-22</sup>. These deficiencies hampered the use of this CML mouse model in a functional assay. Through modification of the original bone marrow transduction protocol, an efficient and rapid induction of a CML-like mouse model was established<sup>23,24</sup>. In this improved system, in order to enrich hematopoietic stem cells, donor mice were primed by intravenous injection with 5-fluorouracil (5-FU) 4 days before collecting bone marrow cells<sup>23,24</sup>. Cells were cultured with cytokines including interleukin-3 (IL-3), IL-6, and stem cell factor (SCF)<sup>23,24</sup>. To increase transduction efficiency, virus and cells were co-sedimented. In our lab, using this model, we are able to obtain 100% incidence of the CML-like syndrome in recipient mice by 2-4 weeks after transfer of p210<sup>BCR-ABL</sup>-transduced bone marrow cells<sup>23</sup>.

Therefore, this consistent and rapid CML disease model allows us to study the molecular mechanisms of CML development and the maintenance of CML stem cells.

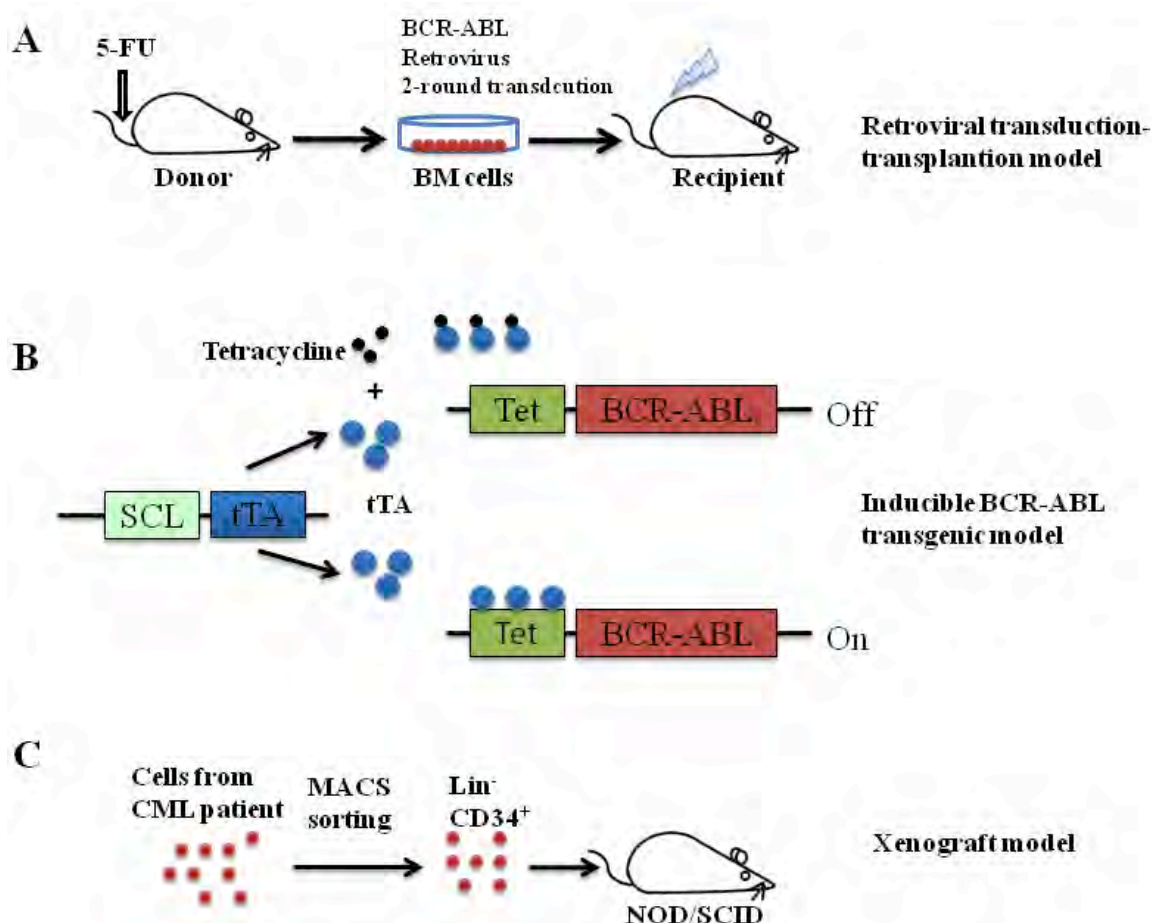
The transgenic mouse is an alternative and useful approach to investigate BCR-ABL induced leukemia *in vivo*. Several BCR-ABL transgenic mice strains have been created using different regulatory elements to drive BCR-ABL expression<sup>25,26</sup>. The transgenic mice expressing p210BCR-ABL driven by the metallothionein (MT) promoter developed T-cell leukemia<sup>25</sup>. Huettner et al. generated an inducible transgenic strain in which BCR-ABL expression was controlled by tetracycline transactivator (tTA) through the mammary tumor virus long terminal repeat (MMTV-LTR)<sup>26</sup>. Withdrawal of tetracycline administration in this transgenic strain allowed expression of BCR-ABL and resulted in the development of acute B-cell leukemia in 100% of the mice within 3-6 weeks<sup>26</sup>. These studies demonstrate that the phenotype of BCR-ABL transgenic mice is dependent on the expression pattern and cell-type specificity of the regulatory elements: in the MT-BCR-ABL transgenic model, as the tumorigenicity of BCR-ABL chimeric protein is restricted to the hematopoietic tissues *in vivo*, the MT promoter only drives BCR-ABL expression in the hematopoietic tissues, thymus and spleen, thus accounting for the T-cell phenotype<sup>25</sup>; in the MMTV-LTR/tTA-BCR-ABL inducible model, the MMTV-LTR directs expression of tTA to early B-cell progenitors within murine bone marrow, thus accounting for the B-cell phenotype<sup>26</sup>. Given that CML is a stem cell disorder, it is reasonable that the regulatory elements derived from HSC-specific genes should cause a more accurate model of human CML. The *SCL* (stem cell leukemia) gene encodes a basic helix-loop-helix transcription factor, which mainly expresses in



hematopoietic cells, endothelial cells, and embryonic skeleton, and is critical for cell fate determination and differentiation<sup>27-29</sup>. An enhancer fragment within the 3' region of *SCL* has been identified to restrict expression of the LacZ transgene in adult HSCs, progenitor cells and megakaryocytes<sup>30,31</sup>. Based on these findings, Koschmieder et al. generated the SCLtTA/TRE-BCR-ABL transgenic model<sup>32</sup> (Figure 2B), in which tTA was driven by the SCL 3' enhancer. Induction of BCR-ABL resulted in neutrophilia, splenomegaly, and myeloid invasion, which models the major features of human CML disease<sup>32</sup>, and also altered the biology of stem cell and progenitor cell populations<sup>33</sup>. Therefore, this inducible transgenic model represents a valuable alternative for CML study.

The ideal model for investigating CML is the xenograft model in which primary cells from human CML patients are transplanted into SCID mice to mimic human CML disease (Figure 2C). However, many efforts have proved that this model is more difficult to establish due to the failure of engraftment of CML cells<sup>34,35</sup>. This failure of engraftment may be a result of the rare CML stem cells in most chronic phase CML patients, or the lack of a suitable immunodeficient mouse strain. Recently, Eisterer et al<sup>36</sup> used only patient samples in which the long-term culture-initiating cells were predominantly Ph<sup>+</sup>, and then transplanted Lin<sup>-</sup> or Lin<sup>-</sup>CD34<sup>+</sup> cells from these samples into sublethally irradiated NOD/SCID and NOD/SCID- $\beta$ 2microglobulin<sup>-/-</sup> mice<sup>36</sup>. The average engraftment level ranged from 0.02% to 10% after transplantation, and the highest frequencies of human cells were typically seen after 3 weeks<sup>36</sup>. In our lab, we sorted Lin<sup>-</sup>CD34<sup>+</sup> bone marrow cells from human CML patients in the chronic phase and transplanted these cells into NOD/SCID/IL2R $\gamma$ <sup>-/-</sup> mice which supports engraftment of

human CD34<sup>+</sup> hematopoietic stem cells<sup>37,38</sup>. The highest frequencies (about 5-10%) of human cells (human CD45<sup>+</sup>) were typically observed after 2 weeks, and about 1% of human cells were persistent for 2-3 months in most recipients (unpublished data). Therefore, these findings demonstrate the efficacy of the xenograft model of human CML.



**Figure 2. CML mouse models.** (A) Retroviral transduction/transplantation model. Bone marrow cells from 5-FU (200mg/kg) pretreated donor mice are transduced with *BCR-ABL* retrovirus twice. These cells are then transplanted into lethally irradiated recipients for induction of CML<sup>23</sup>. (B) *BCR-ABL* transgenic model. The SCL 3' enhancer drives the expression of tetracycline dependent transcription factor (tTA) in hematopoietic stem/progenitor cells<sup>31</sup>. The presence of tetracycline blocks the binding of tTA to the *Tet* promoter, thus shutting off *BCR-ABL* expression. Conversely, tTA binding to the *Tet* promoter in the absence of tetracycline activates transcription of *BCR-ABL* in

hematopoietic stem/progenitor cells, leading to the induction of CML disease<sup>33</sup>. (C) Xenograft model. Lin<sup>-</sup>CD34<sup>+</sup> cells from human CML patients are sorted by magnetic-activated cell sorting (MACS) and injected into NOD/SCID mice.

#### 4. BCR-ABL tyrosine kinase inhibitors

The discovery of the molecular basis of CML provided the foundation for designing therapeutic drugs and led to the development of the BCR-ABL tyrosine kinase inhibitor (TKI), imatinib, the first TKI found to be highly effective in CML treatment<sup>39-41</sup>. Imatinib can bind to the ABL kinase domain through forming hydrogen bonds, and impair ATP binding, which block the substrate activation<sup>42</sup>. Therefore, targeting therapy with imatinib induces a complete hematologic and cytogenetic response in more than 80% of chronic phase CML patients<sup>43</sup>. However, resistance to TKI has been frequently observed, especially in patients with advanced-stage disease<sup>44</sup>. One critical reason is the occurrence of point mutations in the kinase domain of BCR-ABL<sup>45</sup>. Using a PCR strategy to determine the sequence of the ATP binding pocket and activation loop of the kinase domain of ABL coming from relapsed patient samples, Gorre et al. identified a single nucleotide C to T change, which results in a threonine to isoleucine substitution at position 315, that is, the well-known T315I mutation<sup>44</sup>. This substitution of threonine precludes the formation of a hydrogen bond with TKI but does not interfere with ATP binding<sup>45</sup>. To date, more than 50 point mutations have been found, including mutations of contact residues, mutations in the ATP binding loop and the regulatory motifs<sup>46</sup>. These mutations prevent ABL from adopting the specific conformation required for high-affinity imatinib binding<sup>46</sup>.

To override this resistance, secondary-generation TKIs, such as dasatinib and nilotinib, have been developed. Dasatinib is a dual ABL and Src family kinases (SFKs) kinase inhibitor<sup>47</sup>. Previous studies proved that dasatinib overcame imatinib resistance conferred by most ABL kinase mutations, as dasatinib is able to bind both the inactive and active states of ABL with higher affinity<sup>48-51</sup>. Similarly, nilotinib has a higher affinity for the inactive conformation of BCR-ABL by 20 to 30 fold<sup>52</sup>, which makes it highly efficacious in patients with CML following failure of imatinib therapy<sup>53</sup>. However, both inhibitors cannot inhibit the gatekeeper residue mutant T315I. With the development of structural biology and computational biology, an alternative strategy to target other BCR-ABL motifs that are remote from the kinase domain has been developed<sup>54-56</sup>. This strategy would be unaffected by kinase mutations that confer the ability of TKI-resistance. Through a high-throughput screen, GNF-2, a highly selective non-ATP competitive inhibitor of BCR-ABL, was identified<sup>54</sup>. A recent study using nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography confirmed that GNF-2 bound to the C-terminal myristoyl pocket of ABL, leading to changes in the structural dynamics of the ATP-binding site<sup>55</sup>. GNF-5, an analogue of GNF-2 with an improved pharmacokinetic feature, can act cooperatively with an ATP competitive inhibitor to inhibit both wild type and T315I BCR-ABL activity<sup>55</sup>. Additionally, using structure-based drug design, Chan et al developed an inhibitor, DCC-2036, which potently inhibits both unphosphorylated and phosphorylated ABL1 by inducing an inactive conformation<sup>56</sup>. This inhibitor exhibited efficacy against the T315I mutant in

vivo<sup>56</sup>. The development of these inhibitors provides the possibility of overcoming TKI resistance caused by kinase mutations.

## **5. Beyond TKIs---cancer stem cells in chronic myeloid leukemia**

Although the majority of CML patients achieve a complete hematological response, and up to 90% can achieve a complete cytogenetic response to TKI therapy with at least 2 years follow-up<sup>49,50,57,58</sup>, clinical relapse eventually occurs once treatment is interrupted<sup>59</sup>. Additionally, only a very low percentage of complete molecular response is achieved from therapy with imatinib, or new generation TKIs<sup>48,53,60</sup>, which suggests that a population of residual malignant cells is unaffected by TKIs. Now, it is accepted that these TKI-refractory malignant cells are leukemia stem cells (LSCs). The existence of cancer stem cells (CSCs) in CML has been experimentally proved in a series of studies<sup>61-63</sup>. Using carboxy-fluorescein diacetate succinimidyl diester (CFSE) to track cell division, Graham et al showed that imatinib treatment eradicated almost all the dividing CD34<sup>+</sup> cells from patients with CML in the chronic phase, leaving behind the non-proliferating quiescent cells, implying that quiescent CML stem cells are insensitive to imatinib<sup>62</sup>. Further study demonstrated that Ph<sup>+</sup>CD34<sup>+</sup> cells persisted in CML patients who achieved complete cytogenetic response (CCR) with imatinib treatment<sup>61</sup>, and analysis using colony forming cell (CFC) assay and long-term bone marrow culture-initiating cells (LTC-IC) confirmed that these cells retained primitive progenitor ability<sup>61</sup>. In our lab, using the retroviral transduction/transplantation CML mouse model, we found that treatment with imatinib and the secondary generation of TKI, dasatinib, dramatically

prolonged the survival of CML mice; however, all treated CML mice eventually died of this disease<sup>63</sup>. Fluorescence-activated cell sorting (FACS) analysis showed that TKIs reduced the numbers of BCR-ABL-expressing HSCs but failed to eliminate these cells completely in CML mice<sup>63</sup>. A recent study also demonstrated that targeting BCR-ABL could not eliminate CML stem cells, although drug treatment inhibited BCR-ABL activity and killed the proliferating CML progenitor cells<sup>64</sup>. Together these studies indicate TKIs cannot eradicate LSCs, which forces us to reassess the notion of LSCs and their role in the disease, in order to understand the molecular mechanisms of LSC maintenance and to design new strategies to eradicate them.

## **6. Cancer stem cell and chronic myeloid leukemia stem cell**

The cancer stem cell model is not a new idea<sup>65</sup>, but it has attracted broad interest recently due to its promising clinical implications in cancer therapy. Like normal stem cells, cancer stem cells can be defined as a specific cell population inside cancer that can self-renew and has the ability to initiate cancer development<sup>65-68</sup>. Bonnet and Dick first identified and characterized LSCs from human AML samples<sup>69</sup>. They isolated CD34<sup>+</sup>CD38<sup>-</sup> cells and transplanted them into NOD/SCID mice. They found that these cells could not only initiate AML in NOD/SCID mice but also differentiate in vivo into leukemic blasts<sup>69</sup>. More importantly, serial transplantation demonstrated that these cells had a capacity to self-renew and transferred AML disease into secondary recipients<sup>69</sup>. Therefore, this study showed for the first time that LSCs were exclusively CD34<sup>+</sup>CD38<sup>-</sup> cells, which have the same cell-surface phenotype as normal human primitive cells.

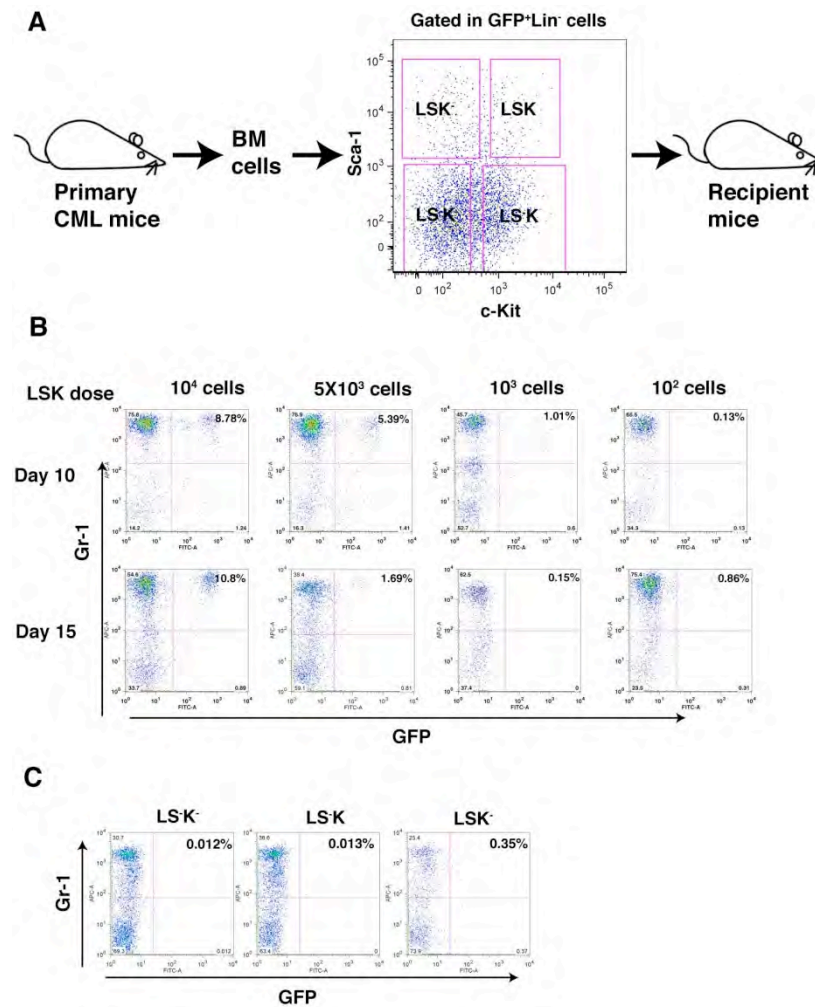
Subsequently, similar approaches were applied to solid tumors. Cancer stem cells have been identified in many kinds of solid tumors, including breast<sup>70</sup>, brain<sup>71</sup>, pancreas<sup>72</sup>, colon<sup>73</sup>, lung<sup>74</sup>, and prostate<sup>75</sup>. These studies indicate that CSCs are a rare phenotypically distinct subpopulation of cancer cells in most of cancers, and these rare CSCs represent the driving force of tumor malignancy. Therefore, targeting CSCs might be an attractive and potentially effective strategy for cancer treatment. These types of therapies would require an understanding of the molecular mechanisms of CSC self-renewal and survival. Considerable evidence shows that CSCs and normal stem cells share similar mechanisms for self-renewal and survival, and many pathways, such as Wnt- $\beta$ -catenin and hedgehog pathways<sup>76,77</sup>.

Many efforts have been undertaken to identify cancer stem cells in CML. The myeloid progenitors, common myeloid progenitor (CMP) and granulocyte-macrophage progenitors (GMP), transduced with BCR-ABL retrovirus did not serially replat, indicating these cells lack the ability of self-renewal<sup>78</sup>. Using the SCLtTA/TRE-BCR-ABL transgenic mouse model, Reynaud et al confirmed that BCR-ABL-expressing progenitors including CMP, GMP and common lymphoid progenitor (CLP), failed to transfer CML disease into secondary recipients<sup>79</sup>. Therefore, these data demonstrate that BCR-ABL cannot confer properties of self-renewal to committed myeloid progenitors. To identify CML stem cells, we tested whether BCR-ABL-expressing HSCs function as LSCs. Using a BCR-ABL induced CML mouse model, we sorted BCR-ABL expressing Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) cells from primary CML mice and transplanted them into lethally irradiated secondary recipients, and found that these BCR-ABL-expressing LSK cells

induced CML disease in the secondary recipients<sup>63</sup>, suggesting that BCR-ABL expressing LSK cells function as LSCs. We further characterized the ability of this stem cell population to transfer CML to secondary recipient mice. We divided BCR-ABL-expressing (GFP<sup>+</sup>) Lin<sup>-</sup> cells into four populations: Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>-</sup> (LSK<sup>-</sup>), Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK), Lin<sup>-</sup>Sca-1<sup>-</sup>c-Kit<sup>-</sup> (LS<sup>-</sup>K<sup>-</sup>), and Lin<sup>-</sup>Sca-1<sup>-</sup>c-Kit<sup>+</sup> (LS<sup>-</sup>K) (Figure 3A). We serially diluted sorted GFP<sup>+</sup>LSK cells from CML mice and transplanted 1x10<sup>4</sup>, 5x10<sup>3</sup>, 1x10<sup>3</sup> or 1x10<sup>2</sup> cells into a lethally irradiated mice along with 2x10<sup>5</sup> normal bone marrow cells. FACS analysis showed that myeloid leukemia cells (GFP<sup>+</sup>Gr-1<sup>+</sup>) were detected in the peripheral blood of mice receiving 1x10<sup>4</sup>, 5x10<sup>3</sup> or 1x10<sup>3</sup> GFP<sup>+</sup>LSK cells at 10 days after BMT, whereas almost no leukemia cells were found in mice receiving 1x10<sup>2</sup> GFP<sup>+</sup>LSK cells (Figure 3B). At day 15 after bone marrow transplantation (BMT), leukemia cells almost disappeared from mice receiving 1x10<sup>3</sup> GFP<sup>+</sup>LSK cells (Figure 3B). Eventually, mice receiving 1x10<sup>4</sup> (100% of the mice) or 5x10<sup>3</sup> (50% of the mice) GFP<sup>+</sup>LSK cells developed CML, and lower cell doses (1x10<sup>3</sup> or 1x10<sup>2</sup>) did not induce CML (Figure 3B). We also tested the other GFP<sup>+</sup> cell populations (LS<sup>-</sup>K, LSK<sup>-</sup>, and LS<sup>-</sup>K cells) for their ability to induce CML in secondary recipient mice. We transplanted 3x10<sup>4</sup> cells along with 2x10<sup>5</sup> normal bone marrow cells into lethally irradiated mice and found that leukemia cells were barely detectable in peripheral blood of recipient mice (Figure 3C), indicating these populations are incapable of inducing leukemia. Similarly, Naka et al. also confirmed that BCR-ABL-expressing LSK cells from CML mice could efficiently induce CML development in recipient mice. In contrast, no other CML cell populations expressing differentiation markers induced CML<sup>80</sup>. Therefore, these studies



suggest that BCR-ABL-expressing LSK cells function as CML LSCs, which exhibit characteristic similar to those of normal HSCs. The identification of CML LSCs allows us to understand the mechanisms by which CML stem cells survive and maintain themselves, which will contribute to the design of new strategies aimed at targeting CML stem cells as a curative therapy for CML.



**Figure 3. Identification of chronic myeloid leukemia stem cells.** (A) Four populations of cells (LSK<sup>-</sup>; LSK; LSK<sup>-</sup>; LSK) were sorted from BCR-ABL induced CML mice and transplanted into lethally irradiated recipients at different doses. (B and C) Only LSK cells can function as LSCs and transfer CML disease into secondary recipients.

## 7. LSCs are independent of BCR-ABL kinase activity

Resistance to TKIs by CML stem cells is a common mechanism among nearly all CML patients<sup>62,63</sup>. As mentioned earlier, primitive Ph<sup>+</sup>CD34<sup>+</sup> cells from CML patients exhibit insensitivity to TKIs<sup>62</sup>. Understanding the mechanisms by which LSCs survive under treatment with TKIs will be critical to designing strategies targeting LSCs. However, it is still unclear why and how LSCs in CML evade TKIs. The development of BCR-ABL mutations renders resistance, but resistance to TKIs is frequently present in the absence of a detectable mutation in BCR-ABL<sup>64</sup>. Therefore the most interesting characteristic of the resistant CML cells is its relationship with BCR-ABL.

It is widely accepted that Ph<sup>+</sup> leukemia cells are addicted to BCR-ABL kinase activity, and BCR-ABL activates numerous downstream signaling pathways, such as RAS, phosphoinositide 3-kinases (PI3K), and signal transducer and activator of transcription (STAT), which are critical in BCR-ABL induced leukemogenesis through regulating cell proliferation, survival and differentiation<sup>81-83</sup>. However, it remains unclear whether LSCs are dependent or independent of BCR-ABL kinase activity. Recent studies indicate that LSCs of CML are independent of BCR-ABL kinase activity<sup>64,84</sup>. Using multiple complementary methods, including immunocytochemistry, immunoblot and LTC-IC assay, Druker and colleagues confirmed this idea in *in vitro* cultured cells<sup>64</sup>. Intracellular phosphotyrosine levels determined by FACS analysis showed similar degrees of BCR-ABL inhibition by TKIs in different immature cell subtypes, including CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> populations from newly diagnosed CML patients<sup>64</sup>. Consistently, immunoblot analysis showed that imatinib equivalently completely

inhibited the phosphorylation of CRKL, a BCR-ABL specific substrate, and STAT5 in CML stem and progenitor cells<sup>64</sup>. Further, phospho-CRKL immunoblots of sorted quiescent (Ki67<sup>-</sup>) and cycling (Ki67<sup>+</sup>) cells showed equal inhibition of BCR-ABL activity in both fractions<sup>64</sup>. These data demonstrated that imatinib equally inhibits BCR-ABL in CML stem cells, progenitor cells, and quiescent cells. This inhibition is continuous and does not select a subpopulation with innate resistance, as a similar distribution of cell types in untreated versus imatinib treated cultures was observed<sup>64</sup>. Finally, when cytokines were added, these cells remained capable of in vitro proliferation despite continuous imatinib treatment, and an LIT-IC assay showed quiescent primitive cells were only slightly reduced by imatinib treatment<sup>64</sup>. Therefore, these data suggested that the survival of primitive CML cells was independent of BCR-ABL activity. This study also excluded the possibility that drug efflux/influx affects TKI sensitivity of CML stem cells, as comparable inhibition was observed in CML stem cells and progenitors<sup>64</sup>.

Consistently, a study from another group also indicated that CML stem cells are not dependent on BCR-ABL kinase activity for their survival<sup>84</sup>. Human CML CD34<sup>+</sup> cells were cultured in serum-free SFM media and treated with dasatinib. Immunoblot analysis showed the complete inhibition of phospho-CRKL by dasatinib treatment<sup>84</sup>, suggesting the inhibition of BCR-ABL kinase activity. However, although fewer cells (10% of input) were achieved after 12-days of treatment, these cells displayed similar proliferation abilities compared to untreated cells when cytokines were supplied<sup>84</sup>. They also retained their self-renewal ability<sup>84</sup>. Together, these results suggest that CML stem cells utilize

signaling pathways that are independent of BCR-ABL kinase activity for their maintenance and survival.

This notion raises various questions related to what the BCR-ABL kinase independent pathways are and how they regulate the survival of CML stem cells. In our lab, we have found several pathways that are independent of BCR-ABL kinase activity<sup>85,86</sup>.

1) BCR-ABL/Src pathway in B-ALL. Src family kinases are critical for the cellular transformation by BCR-ABL<sup>87,88</sup>. In B-lymphoid cells imatinib markedly inhibited BCR-ABL kinase activity but did not result in a decrease in Src activation<sup>86</sup>. Given the fact that BCR-ABL activates Src kinases, these results suggest that the activation of Src kinases by BCR-ABL is independent of its kinase activity<sup>63</sup>. Using the retroviral transduction/transplantation CML mouse model discussed above, we find another Src kinase, B lymphoid kinase (*Blk*), is significantly down-regulated by BCR-ABL in LSCs, and imatinib treatment fails to restore this down-regulation (the detailed information will be described in CHAPTER III), suggesting that the down-regulation of *Blk* expression in LSCs is independent of BCR-ABL kinase activity.

2) BCR-ABL/Alox5 pathway. Another example is arachidonate 5-lipoxygenase (*Alox5*), which is dramatically induced by BCR-ABL in CML LSCs<sup>85</sup>; however, inhibition of BCR-ABL kinase by imatinib cannot suppress this up-regulation of *Alox5*<sup>85</sup>, suggesting that *Alox5* expression in LSCs is independent of BCR-ABL kinase activity.

Therefore, these findings of BCR-ABL kinase independent pathways provide an alternative explanation for the resistance of LSCs to TKIs. It is deduced from these

findings that completely different strategies, such as targeting the pathways of stem cell self-renewal or targeting the BCR-ABL kinase independent pathways, are needed to eliminate LSCs.

## **8. Molecular pathways regulating the maintenance of LSCs**

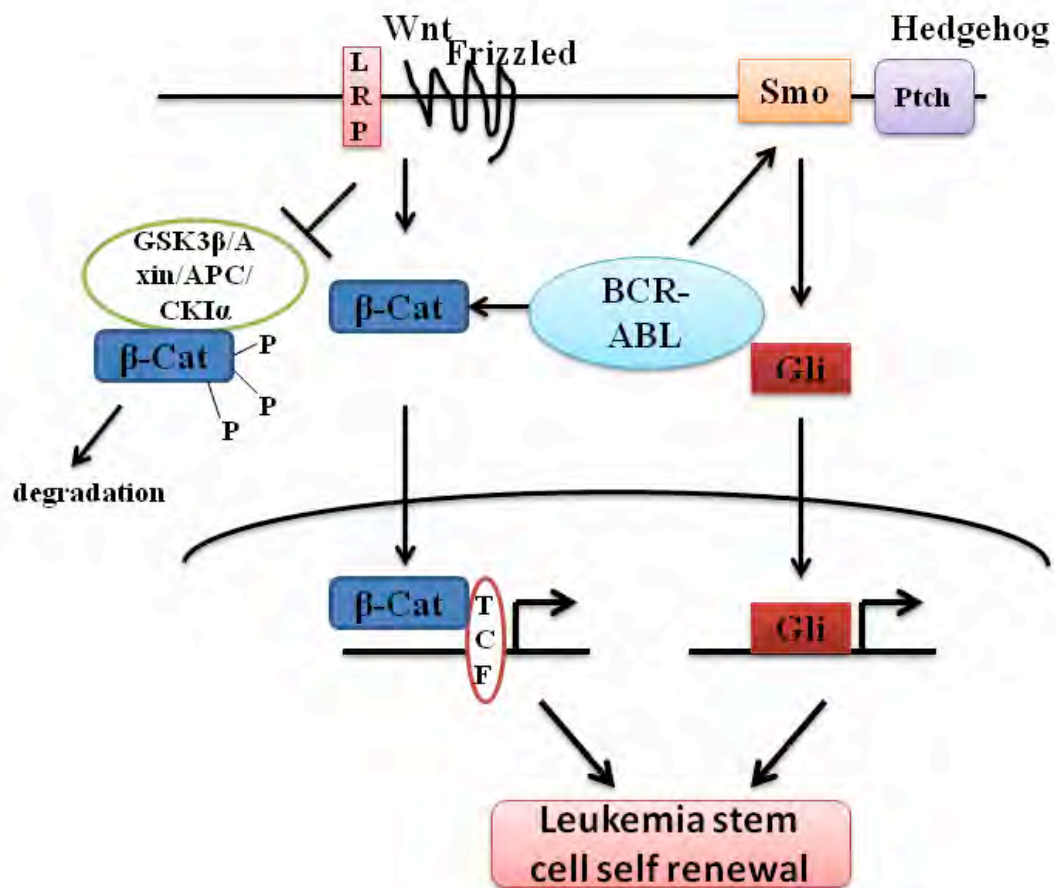
The finding that CML LSCs are resistant to TKI treatment leads us to ponder why LSCs evade BCR-ABL kinase inhibitor treatment. What is the nature of LSCs? How do LSCs survive whereas other CML cells are eliminated by TKI therapy? To answer these questions requires a full understanding of the biology of LSCs. As LSCs share functional properties (self-renewal, and pluripotency) with normal HSCs<sup>65,89,90</sup>, it is reasonable to consider that signaling pathways involved in the regulation of normal stem cells should also be essential for LSCs. As described in the following section, many studies demonstrate that Wnt/ $\beta$ -catenin<sup>91</sup>, hedgehog<sup>92</sup>, TGF-FOXO<sup>80</sup>, BCL6<sup>93</sup>, and HIF1 $\alpha$ <sup>94</sup> signaling pathways that are essential for normal HSC self-renewal are also required for CML LSCs.

### **The Wnt/ $\beta$ -catenin pathway**

The Wnt/ $\beta$ -catenin signaling pathway, an important developmental pathway, is necessary for stem cell self-renewal<sup>95</sup>. The canonical Wnt pathway is initiated by binding of Wnt ligands to their receptors Frizzled and LRP5/6<sup>95</sup>. This consequently destabilizes the multiprotein destruction complex that contains Axin, glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), casein kinase 1 (CK1) and adenomatous polyposis coli (APC)<sup>95</sup>. This disrupts

the binding of GSK3 $\beta$  with  $\beta$ -catenin and leads to the dephosphorylation of  $\beta$ -catenin<sup>95</sup>. Unphosphorylated  $\beta$ -catenin accumulates and translocates into the nucleus, where it interacts with the lymphoid enhancer factor/T-cell factor transcription factors and regulates the transcription of genes such as *c-myc* and *cyclin D1*<sup>95-98</sup>. Dysregulated Wnt/ $\beta$ -catenin signaling has been found in certain human leukemia and LSCs<sup>98,99</sup> (Figure 4). In acute myeloid leukemia, activated Wnt/ $\beta$ -catenin signaling is required for the self-renewal of LSCs derived from HSCs or more differentiated granulocyte-macrophage progenitors (GMP) induced either by coexpression of *Hoxa9* and *Meis1a* or by MLL-AF9<sup>99</sup>. Similarly, self-renewal of BCR-ABL<sup>+</sup> CML cells is also sustained by a nuclear activated serine/threonine unphosphorylated  $\beta$ -catenin<sup>100</sup>. GMP cells from CML patients in blast crisis have accumulated nuclear  $\beta$ -catenin and exhibit enhanced self-renewal ability *in vitro*<sup>100</sup>. Using a CML mouse model, Zhao et al demonstrate that loss of  $\beta$ -catenin impairs the development of BCR-ABL induced CML due to a defect in cancer stem cell self-renewal without  $\beta$ -catenin<sup>91</sup>. The mechanism underlying  $\beta$ -catenin deregulation in CML has been investigated. BCR-ABL physically interacts with  $\beta$ -catenin, and phosphorylates  $\beta$ -catenin at tyrosine residues at position 486 and 654. This subsequently induces  $\beta$ -catenin stabilization by interrupting binding to Axin<sup>101</sup>. Unlike the essential role of  $\beta$ -catenin in LSCs<sup>91</sup>, the  $\beta$ -catenin pathway function in adult HSCs is controversial. Activation of Wnt signaling by retrovirus-mediated expression of constitutively active  $\beta$ -catenin in HSCs or exposure to purified Wnt3a can increase self-renewal of murine HSCs *in vitro* and enhance their ability to reconstitute the hematopoietic system *in vivo*<sup>102,103</sup>. However, several studies demonstrate that deficiency

of  $\beta$ -catenin,  $\gamma$ -catenin, or both, does not perturb hematopoiesis<sup>104,105</sup>, suggesting that Wnt-dependent effects on adult HSCs might be subtle. Given the critical role of Wnt/ $\beta$ -catenin pathway in LSCs, a drug that can target this pathway could offer a new treatment approach for targeting LSCs. A recent study confirmed the feasibility of this approach, as treatment with indomethacin, a reversible COX inhibitor that blocks activation of the Wnt pathway by inhibiting  $\beta$ -catenin expression, reduced LSCs in AML mice<sup>99</sup>.



**Figure 4.** Wnt/ $\beta$ -catenin and hedgehog pathway in leukemia stem cells.

## The hedgehog pathway

Hedgehog (Hh) is a developmental signaling pathway in which Hh ligands, including Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh), bind the seven-transmembrane domain receptor Patched (Ptch)<sup>106</sup>. This leads to the release of Ptch binding from Smoothened (Smo)<sup>107</sup>. Subsequently, Smo activates Hh pathway target genes like *Gli1*, *Ptch1*, *cyclin D1*, and *Bcl2* using the Gli transcription factors<sup>107</sup> (Figure 4). The role of Hh signaling in normal and malignant hematopoiesis is controversial. To understand the role of Hh signaling in normal HSCs, Hofmann et al<sup>108</sup> and Gao et al<sup>109</sup> in parallel studies examined the effects of the conditional *Smo* deletion in the hematopoietic system. They demonstrated that deletion of *Smo* had no apparent effect on adult HSC self-renewal and function, including colony-forming potential and long-term repopulating activity in a competitive repopulation assay, suggesting that Hh signaling is dispensable for normal HSC function<sup>108,109</sup>. In leukemogenesis, deletion of *Smo* does not affect MLL-AF9 induced AML, which is shown by a similar *in vitro* replating potential and *in vivo* leukemia development between MLL-AF9 transduced WT and *Smo*<sup>-/-</sup> cells<sup>108</sup>. However, these findings do not exclude the possibility that Hh signaling is required for other types of leukemia. Most attention has been focused on the role of Hh signaling in CML. Interestingly, Hh signaling is activated in LSCs of CML, which show up-regulation of *Smo*<sup>92,110</sup>. Loss of *Smo* impaired BCR-ABL induced CML development and caused depletion of CML LSCs, as LSCs failed to transfer CML disease into secondary recipients<sup>92,110</sup>. These findings suggest that Hh signaling is essential for maintenance of CML LSCs (Figure 4). Pharmacological inhibition of Hh signaling with



cyclopamine reduced the colony-forming potential of LSCs and increased the time to relapse after the end of treatment<sup>110</sup>. Importantly, LSCs with mutated BCR-ABL bearing the mutation T315I were also sensitive to Smo inhibition<sup>110</sup>. Given the fact that Hh signaling is dispensable for normal HSCs<sup>109</sup>, it is reasonable to think that combining targeting of this essential pathway and administration of TKIs might be an effective treatment strategy to specifically reduce the LSC pool in CML.

### **The FoxO pathway**

The forkhead O transcription factors (FoxO1, FoxO3, FoxO4, FoxO6), as the important downstream targets of PI3K-Akt signaling, are involved in various cellular processes, such as cell cycle arrest, differentiation, apoptosis, stress resistance and metabolism<sup>111</sup>. Akt directly phosphorylates FoxO1, FoxO3 and FoxO4<sup>112,113</sup>. This leads to the binding of the 14-3-3 chaperone proteins to FoxOs that interfere with the DNA binding domain of FoxOs and induce the export of FoxOs from the nucleus to the cytoplasm<sup>112,113</sup>. This translocation of FoxOs results in their proteasomal degradation in the cytoplasm<sup>112,113</sup>. Therefore, while FoxO inactivation by Akt increases cell survival, proliferation and stress sensitivity, activation of FoxO residing in the nucleus causes cell cycle arrest by regulating the expression of p27, p21, cyclin D, cyclin G2, and cyclin B<sup>114</sup>. It is widely accepted that BCR-ABL activates the PI3K-Akt pathway, leading to inactivation of FoxO transcription factors that lead to increases in CML cell proliferation and survival<sup>115</sup>. Unexpectedly, using a retroviral transduction/transplantation CML mouse model, Naka et al. identified a converse role for Akt-FoxO in LSCs of CML<sup>80</sup>. Unlike

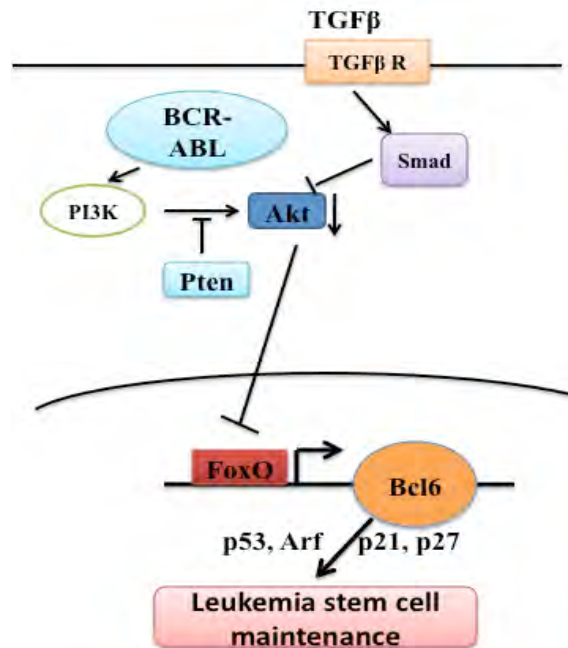
leukemia progenitor cells showing enhanced Akt phosphorylation and higher cytoplasmic localization of FoxO3a, CML LSCs exhibited decreased Akt phosphorylation and enriched nuclear localization of FoxO3a<sup>80</sup> (Figure 5). Furthermore, FoxO3a deletion impaired CML development in a serial transplantation assay, suggesting FoxO3a is essential for the long-term maintenance of CML LSCs<sup>80</sup>. Consistently, FoxO also has a surprisingly essential role in the maintenance of the undifferentiated status of LSCs in AML<sup>116</sup>. LSCs from MLL-AF9 induced AML have decreased Akt activation and active FoxOs. Deletion of FoxO1/FoxO3/FoxO4 decreased colony-forming ability, promoted myeloid maturation and diminished LSC function *in vivo*<sup>116</sup>. Moreover, about 40% of AML patients displayed active FoxOs, which is consistent with a previous observation that high expression of FoxO3 is associated with adverse prognosis in AMLs with normal cytogenetics<sup>117</sup>. These data demonstrate an opposite role for FoxOs in LSCs that is counter to their established function as tumor suppressors in many cancers. The study of FoxOs in normal HSCs might provide key insight into what is observed in LSCs. Deletion of FoxO1/FoxO3/FoxO4 in the adult hematopoietic system led to a marked decrease of HSC numbers and resulted in the defective long-term reconstitution ability<sup>114</sup>. The effects of FoxOs on HSCs correlated with the modulations of their target genes in the cell cycle, including *p21*, *p27*, *cyclin D2*, and *cyclin G2*<sup>114</sup>. FoxO deficiency also caused increased apoptosis of HSCs. Therefore, FoxOs are required for the maintenance of HSCs. Further, Naka et al also demonstrated that the TGF- $\beta$ -Smad2/3 pathway regulated the retention of FoxO3a in the nucleus in CML LSCs, and that TGF- $\beta$  inhibition caused the nuclear exclusion of FoxO3a<sup>80</sup>. A combination of TGF- $\beta$  inhibition and imatinib

treatment led to more efficient depletion of CML in vivo<sup>80</sup>. Thus targeting FoxOs and related signaling pathways might represent a new therapeutic way for eliminating LSCs.

### **The BCL6 pathway**

B cell lymphoma 6 (*BCL6*) is a known proto-oncogene encoding a BTB/POZ-zinc-finger transcriptional repressor<sup>118,119</sup>. It is known that BCL6 is necessary for germinal-center formation and is involved in the pathogenesis of B-cell lymphoma<sup>118</sup>. Therefore, the majority of previous studies about BCL6 focus on B cells and B lymphoma. BCL6 suppresses p53 transcription by binding the promoter of p53 and modulates DNA damage-induced apoptotic responses in germinal-center B cells<sup>119</sup>. Constitutive expression of BCL6 protects B cell lines from apoptosis induced by DNA damage, suggesting that deregulated BCL6 expression contributes to lymphomagenesis in part by functional inactivation of p53<sup>119</sup>. Recent studies indicate that BCL6 plays a critical role in a protective feedback signaling mechanism by LSCs in response to TKI treatment<sup>93,120</sup>. Duy et al. first demonstrated that TKI treatment induced BCL6 up-regulation mediated by decreased phosphorylation of STAT5 and enhanced FoxO4 expression in Ph<sup>+</sup> acute lymphoblastic leukemia (ALL) cells<sup>120</sup>. The target genes of BCL6 in TKI-treated Ph<sup>+</sup> ALL cells, including *p53*, *Arf*, *p21*, and *p27*, were identified by a ChIP on chip and comparative gene expression assay<sup>120</sup> (Figure 5). Furthermore, *BCL6*<sup>-/-</sup> BCR-ABL-transduced B lymphoblast failed to induce leukemia in immunodeficient mice, which was suggestive of a defect in the self-renewal of *BCL6*<sup>-/-</sup> LSCs<sup>120</sup>. Interestingly, deletion of *BCL6* restored the sensitivity of B-ALL cells to imatinib,

indicating a protective role for BCL6 in LSCs of Ph<sup>+</sup> ALL treated with TKIs<sup>120</sup>. Muschen and colleagues also extended their findings to LSCs in CML<sup>93</sup>. They demonstrated that human primary CD34<sup>+</sup> CML cells exhibited enhanced BCL6 expression upon TKI treatment and that the Pten-Akt-FoxO pathway was responsible for this up-regulation of BCL6<sup>93</sup>, which is validated by our recent study<sup>121</sup>. We found that *Pten* was down-regulated by BCR-ABL, conditional deletion of Pten accelerated CML development, and overexpression of Pten delayed CML development because of the impaired function of LSCs<sup>121</sup>. Additionally, deletion of *BCL6* impaired the colony-forming potential in vitro of LSCs and failed to induce CML<sup>93</sup>, suggesting BCL6 is required for the maintenance of LSCs in CML. Further, p53 was identified as a key transcriptional target of BCL6 in CML<sup>93</sup>. Most importantly, both studies showed that a combination of TKIs and inhibition of BCL6 by the retro-inverso peptide inhibitor RI-BPI, which blocks the repressor activity of BCL6, effectively eradicated LSCs in CML and Ph<sup>+</sup>ALL<sup>93,120</sup>. These exciting findings regarding BCL6 up-regulation after TKI treatment provide one possible explanation for why and how LSCs persist in patients despite long-term TKI treatment and make it a potential target in TKI resistant LSCs in both CML and Ph<sup>+</sup> ALL.



**Figure 5. Akt-FoxO-Bcl6 pathway in leukemia stem cells.**

### **The hypoxia inducible factor pathway**

HIF1 belongs to the family of basic helix-loop-helix (bHLH) transcription factors<sup>122</sup>. It forms a heterodimer that consists of a constitutively expressed HIF1 $\beta$  subunit and a HIF1 $\alpha$  subunit, and mediates cellular adaptation to hypoxia<sup>122</sup>. The expression of HIF1 $\alpha$  is regulated in multiple ways. Under normoxic conditions, HIF1 $\alpha$  is degraded rapidly, which is triggered by the oxygen-hydroxylation of proline residues 402 and 564 by a proline hydroxylase (PHD)<sup>123</sup>. The hydroxylated HIF1 $\alpha$  is recognized by the Von Hippel-Lindau protein (pVHL), which is the recognition component of an E3 ubiquitin-protein ligase<sup>123,124</sup>. HIF1 $\alpha$  expression is also regulated by growth factors, cytokines and other signaling pathways such as PI3K and MAPK pathways<sup>125</sup>. It is

widely accepted that HIF1 $\alpha$  plays an important role in cancer progression by activating transcriptional programs for self-renewal and multipotency of cancer stem cells<sup>126-128</sup>. Recent studies from our group and another group have added HIF1 $\alpha$  as one of the key pathways in LSCs of hematological malignancies<sup>94</sup>. Wang et al. demonstrated that cancer stem cells in lymphoma from transgenic mice with an insertional mutation of the *Epm2a* gene had a high level of HIF1 $\alpha$  under normoxia<sup>94</sup>. Both knockdown of HIF1 $\alpha$  by shRNA and inhibiting HIF1 $\alpha$  activity by the inhibitor, echinomycin, which blocks HIF1 $\alpha$  DNA binding activity, reduced the colony forming ability *in vitro* and abrogated lymphoma development *in vivo*<sup>94</sup>, suggesting HIF1 $\alpha$  is essential for maintenance of CSCs of lymphoma. Furthermore, similar to the findings in the lymphoma CSCs, higher HIF1 $\alpha$  activity was only observed in CD34<sup>+</sup>CD38<sup>-</sup> LSCs of human AML<sup>94</sup>. Using a xenogeneic mouse model, the authors showed that echinomycin treatment effectively prevented the engraftment of LSCs from AML patients in a serial transplantation assay<sup>94</sup>. To our interest, BCR-ABL induces *HIF1a* expression at the transcriptional level in leukemia cell lines, which is mediated by PI3K and mTOR but not MAPK pathways, as this up-regulation was only inhibited by the addition of either PI3K inhibitor LY294002 or mTOR inhibitor rapamycin<sup>129</sup>. These findings suggest that HIF1 $\alpha$  might contribute to the pathogenesis of CML. Using a retroviral transduction/transplantation CML mouse model, we found, which will be mentioned in CHAPTER II, that an active HIF1 $\alpha$  signaling pathway was maintained in LSCs of CML comparing to normal HSCs. HIF1 $\alpha$ <sup>-/-</sup> LSCs gave rise to less colonies *in vitro* and failed to induce CML disease in the secondary recipients<sup>130</sup>, suggesting that HIF1 $\alpha$  is required for self-renewal of LSCs in CML.

Further, we showed that HIF1 $\alpha$ <sup>-/-</sup> LSCs have enhanced levels of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> and that deletion of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> by lentivirus-mediated shRNA rescued the defective colony-forming potential of LSCs due to the absence of HIF1 $\alpha$ <sup>130</sup>. Importantly, microarray analysis showed significant changes of HIF1 $\alpha$  target genes in HIF1 $\alpha$ <sup>-/-</sup> LSCs, compared to HIF1 $\alpha$ <sup>-/-</sup> HSCs, suggesting that LSCs of CML are more dependent on HIF1 $\alpha$  activity than HSCs<sup>130</sup>. Given the higher specificity and sensitivity of CSCs in lymphoma and human AML to HIF1 $\alpha$  inhibitor echinomycin<sup>94</sup>, it is reasonable to hypothesize that it is possible to eradicate LSCs in CML by targeting HIF1 $\alpha$ , although the efficiency of echinomycin or other HIF1 $\alpha$  inhibitors on LSCs of CML will need to be validated in a future study.

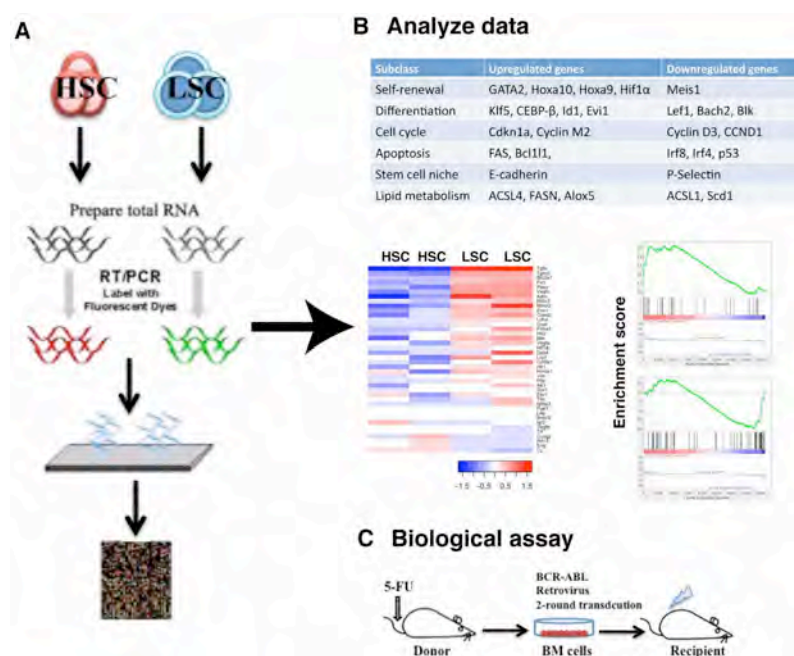
## 9. Current strategies for identifying therapeutic targets in LSCs

Understanding the mechanisms of the survival and self-renewal of LSCs and designing new strategies to target LSC are necessary and critical for curing CML. The identification of the above molecular mechanisms in LSCs makes this goal possible<sup>80,91-94</sup>, however, the critical roles of most of these pathways in normal stem cells limit their utilization for curing CML. Therefore, developing strategies to uniquely target LSCs is crucial. One strategy is to identify agents that specifically eliminate LSCs. The study from Weinberg and colleagues prove the feasibility of this strategy<sup>131</sup>. They screened specific inhibitors of epithelial cancer stem cells using a high-through screen method and found that one compound, salinomycin, selectively killed breast CSCs and inhibited mammary tumor growth through inducing differentiation<sup>131</sup>. Importantly, global gene

expression analyses showed reduced expression of CSC-associated genes after treatment, implying the existence of unique genes that are associated with this specific phenotype of CSCs<sup>131</sup>. In our lab, our strategy is to identify genes that play key roles in LSCs but not their normal stem cell counterparts, as we hypothesize that relative to their normal stem cell counterparts, LSCs utilize a unique molecular network to regulate their functions and that it is possible to identify these genes for developing targeted therapies. For these reasons, comparing the global gene profiling of normal HSCs and LSCs in CML will provide us a straightforward method to identify the key differences between LSCs and their normal counterparts. Towards this goal, using BCR-ABL-induced CML as a disease model, we have performed a microarray to explore the gene expression profiling of CML stem cells (Figure 6A), analyzed these microarray data via bioinformatics methods. Based these information and literatures, we picked up several candidates and assessed the biological functions of candidates using a series of experimental approaches (Figure 6B and C). We have previously identified the *Alox5* gene as a key regulatory gene in LSCs but not normal HSCs<sup>85</sup>. This study proves the principle that it is realistic and feasible to find the specific genes in LSCs. Two more genes, *Blk* and *Scd1*, were identified. *Blk* belongs to the Src family kinases (SFKs). Since our previous studies have indicated that SFKs play dynamic roles in leukemogenesis, this promotes us to further investigate functional role of *Blk* in leukemogenesis using CML mouse model. Interestingly through analyzing our microarray data, we observed that many lipogenesis-related genes were significantly altered in LSCs compared to normal stem cells. *Scd1* is a rate-limiting enzyme involved in converting saturated fatty acids into unsaturated fatty acids.



Therefore we chose *Scd1* as our target gene to study out of hundreds of lipogenesis related candidate genes. The detailed experimental results will be described in CHAPTER III and IV respectively.



**Figure 6. Strategy for understanding the signature of LSCs**

### LSC-specific regulator---Alox5

The *Alox5* gene encoding arachidonate 5-lipoxygenase (5-LO) is involved in numerous physiological and pathological processes, including oxidative stress response, inflammation and cancer<sup>132-139</sup>. 5-LO is responsible for producing leukotrienes, a group of inflammatory substances that cause human asthma<sup>140</sup>. Altered arachidonate metabolism by leukocytes and platelets was reported in association with myeloproliferative disorders almost 30 years ago<sup>141</sup>. Several selective 5-LO inhibitors were found to reduce proliferation of and induce apoptosis of CML cells *in vitro*<sup>142,143</sup>,

although potential off-target effects of these inhibitors were not excluded in these studies. However, the function of *Alox5* in LSCs needs to be tested. Our microarray analysis showed that the *Alox5* gene was upregulated by BCR-ABL in CML LSCs and that this upregulation was not inhibited by imatinib treatment<sup>85</sup>, providing a possible explanation why LSCs are not sensitive to inhibition by BCR-ABL kinase inhibitors<sup>63,64</sup>. Furthermore, recipients of BCR-ABL transduced bone marrow cells from *Alox5*<sup>-/-</sup> donor mice were resistant to the induction of CML by BCR-ABL<sup>85</sup>. FACS analysis of CML cells from the peripheral blood and bone marrow of recipients receiving BCR-ABL-transduced *Alox5*<sup>-/-</sup> donor bone marrow cells showed that myeloid leukemia cells proliferated initially, peaking around 2 weeks, then started to decline, and eventually disappeared after 7 weeks<sup>85</sup>. *Alox5* deficiency mainly affected growth of BCR-ABL-expressing but not non-BCR-ABL-expressing donor bone marrow cells<sup>85</sup>, suggesting that *Alox5* signaling is much more critical for the function of LSCs than for normal HSCs. The effect of *Alox5* on LSC function was further demonstrated and supported by the failure of BCR-ABL-expressing *Alox5*<sup>-/-</sup> bone marrow cells to induce CML in secondary recipient mice<sup>85</sup>. Together, these results suggest that *Alox5* could be a specific target gene in CML LSCs.

### **Src family kinases in leukemogenesis**

The Src family kinases (SFKs) are non-receptor intracellular tyrosine kinases and are comprised of nine members: Src, Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn<sup>144</sup>. SFKs display diverse roles in leukemogenesis<sup>86,145,146,147</sup>. Enhanced phosphorylation levels of

Src have been found in primitive and committed progenitor cells isolated from CML patients<sup>88</sup>, and BCR-ABL interacts with and activates Hck and Lyn in myeloid cells through multiple binding domains<sup>145,87,148</sup>. Our previous data also show that BCR-ABL can activate the SFKs Lyn, Hck and Fgr in B-lymphoid cells<sup>86</sup>. These data suggest that SFKs are involved in the proliferation of BCR-ABL induced leukemia. This is supported by the fact that the activation of Hck phosphorylates Tyr177 in the BCR moiety of BCR-ABL, which promotes the binding of Grb2 leading to the activation of downstream pathways<sup>145</sup>. In addition, Hck also mediates BCR-ABL-dependent activation of STAT5<sup>149</sup>. *Lyn*<sup>-/-</sup> mice are hyperresponsive to myeloid growth factors and develop a myeloproliferative disorder, showing splenomegaly and increased numbers of myeloid progenitors<sup>150</sup>. Loss of two SFKs, Lyn and Hck, results in more HSCs with increased proliferation, reduced apoptosis and skewed differentiation to macrophages. In addition, *Lyn*<sup>-/-</sup>*Hck*<sup>-/-</sup> mice exhibit clinical signs indicative of a myeloproliferative disease by 2 months of age<sup>151</sup>. However, deletion of three SFKs, Lyn, Hck and Fgr, does not affect hematopoiesis, and these mutant mice show no signs of myeloproliferative disease<sup>152</sup>. The role of SFKs in leukemogenesis also has been challenged by recent findings. Our previous data showed that deletion of Lyn, Hck and Fgr is dispensable for BCR-ABL induced CML but is required for B-cell acute lymphoblastic leukemia (B-ALL). These data indicate that SFKs might play a different role in hematopoiesis and leukemogenesis. The studies on another SFK, Blk, mainly focus on B cell development, and its role in leukemogenesis remains unknown. The absence of Blk modestly affects B cell development<sup>153</sup>; however, combined deficiency of the three Src kinase, Blk, Fyn, and

Lyn, impairs pre-B cell receptor mediated NF- $\kappa$ B activation, and results in blocking of B cell development<sup>154</sup>. As will be described in CHAPTER III, we find that Blk plays an inhibitory role in BCR-ABL-induced CML. Deletion of Blk accelerates CML development; conversely, overexpression of Blk suppresses the development of CML through its effects on the function of LSCs. Interestingly, we show that Blk is dispensable for normal hematopoiesis. Our data demonstrate an unexpected and critical role for Blk in leukemia stem cells.

### **Scd1 and leukemogenesis**

Scd is an endoplasmic reticulum enzyme that belongs to a family of  $\Delta$ 9-fatty acid desaturase isoforms. Four mouse Scd isoforms (Scd1-4) and two human Scd isoforms (hScd1 and 5) have been identified<sup>155,156</sup>. Although these isoforms exhibit diverse tissue distribution patterns, they share the same enzymatic function that catalyzes the biosynthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA)<sup>157</sup>. Scd1 expression is detected in almost all tissues with a predominant expression in liver<sup>156,158</sup>. Several transcriptional factors, including liver X receptor (LXR), sterol response element binding protein 1c (SREBP-1c), peroxisome proliferator activated receptor (PPAR), and estrogen receptor, are involved in the regulation of Scd1 expression<sup>155</sup>. Serial biochemical assays showed SREBP1c induces Scd1 expression via binding to the sterol regulatory element (SRE) in the promoter of Scd1<sup>159</sup>. Treatment with the LXR agonist T0901317 induces SREBP-1c expression, suggesting that LXR directly activates SREBP-1 gene transcription, which subsequently induces Scd1 expression<sup>160</sup>.

However, the LXR agonist increases hepatic Scd1 expression in the absence of SREBP-1c, suggesting the ability of LXR to regulate Scd1 also occurs via an SREBP-1c independent pathway. An LXR response element is identified in the promoter of Scd1, which mediates the direct transcriptional regulation of Scd1 by LXR<sup>161</sup>. Additionally, diabetes patients treated with the PPAR gamma agonist, rosiglitazone, show higher Scd activity and expression<sup>162</sup>. Another PPAR gamma agonist, pioglitazone, could also increase the levels of Scd1 mRNA and protein in adipose tissue<sup>162</sup>. However, the exact mechanism of how PPAR regulates Scd1 expression is still unclear, although SREBP-1c seems to mediate this regulation. Furthermore, at the post-translational level, Scd1 can be degraded via an ubiquitin-dependent proteasome mechanism<sup>163</sup>.

Scd1 is involved in regulating metabolic pathways related to preadipocyte differentiation, insulin sensitivity, metabolism, and tumorigenesis<sup>157,164-167</sup>. Deletion of *Scd1* leads to decreased synthesis of lipids, especially triglycerides, and causes the resistance to leptin-deficient-induced and diet-induced obesity via increased insulin sensitivity<sup>168</sup>. *Scd1*<sup>-/-</sup> mice have decreased expression of genes of lipogenesis and increased expression of lipid oxidative genes. Scd1 has been implicated in the pathology of several cancers. Increased Scd expression has been detected in human colonic carcinoma, and inhibition of Scd1 impairs proliferation, survival and invasiveness of lung cancer cells<sup>165</sup>, suggesting that Scd1 plays a supporting role in lung cancer cells. The level of Scd1 expression also correlates with predisposition to liver carcinogenesis<sup>166</sup>. However, prostate carcinoma displays lower Scd expression compared to normal prostate epithelium, suggesting that Scd might play an inhibitory role in cancer<sup>169</sup>. However, the

role of Scd1 in leukemogenesis and hematopoiesis remains unknown, although previous studies suggest that fatty acid metabolism plays a role in leukemogenesis and hematopoiesis<sup>170,171</sup>. Using a BCR-ABL induced chronic myeloid leukemia mouse model, we find that deletion of Scd1 accelerates CML development through affecting the function of LSCs but not normal HSCs and that enforced expression of Scd1 dramatically delays the development of CML<sup>172</sup>. These findings suggest that Scd1 plays a tumor suppressor role in BCR-ABL leukemogenesis. It is known that Scd1 affects insulin signaling. Rahman et al. found that the phosphorylation levels of Akt, insulin receptor (IR) and insulin receptor substrates 1 and 2 (IRS1/2) were elevated in the absence of Scd1<sup>173</sup>. Conversely, stable overexpression of Scd1 in muscle cells decreased the phosphorylation of IRS1 and Akt and was sufficient to impair glucose uptake and insulin signaling. We find that deletion of Scd1 results in higher Akt phosphorylation in CML leukemia cells, and Scd1<sup>-/-</sup> LSCs also display dysregulated Pten, p53 and Bcl2 expression<sup>172</sup>, suggesting these molecular changes downstream of Scd1 might be involved in the regulation of CML development by Scd1. Therefore, our study suggests a novel role for Scd1 in leukemogenesis.

## CHAPTER II

### **HIF1 $\alpha$ is required for survival maintenance of chronic myeloid leukemia stem cells**

The work described in this chapter has been published (Zhang et al., *Blood* 2012 119(11): 2595-2607.

#### **Abstract**

Hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ), a master transcriptional regulator of the cellular and systemic hypoxia response, is essential for the maintenance of self-renewal capacity of normal hematopoietic stem cells (HSCs). It is still unknown whether HIF1 $\alpha$  has a role in survival regulation of leukemia stem cells (LSCs) in chronic myeloid leukemia (CML). Using a mouse model of CML, here we report that HIF1 $\alpha$  plays a crucial role in survival maintenance of LSCs. Deletion of HIF1 $\alpha$  impairs the propagation of CML through impairing cell cycle progression and inducing apoptosis of LSCs. Deletion of HIF1 $\alpha$  results in elevated expression of *p16<sup>Ink4a</sup>* and *p19<sup>Arf</sup>* in LSCs, and knockdown of *p16<sup>Ink4a</sup>* and *p19<sup>Arf</sup>* rescues the defective colony-forming ability of *HIF1 $\alpha$ <sup>-/-</sup>* LSCs. Compared to normal HSCs, LSCs appear to be more dependent on the HIF1 $\alpha$  pathway. Together, these results demonstrate that HIF1 $\alpha$  represents a critical pathway in LSCs and inhibition of the HIF1 $\alpha$  pathway provides a therapeutic strategy for eradicating LSCs in CML.

## Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder associated with the Philadelphia chromosome resulting from a reciprocal translocation between chromosomes 9 and 22. The molecular basis of this chromosomal translocation is the formation of the chimeric BCR-ABL protein that functions as a constitutively activated tyrosine kinase<sup>1,2,174</sup>. Although BCR-ABL kinase inhibitors are highly effective in treating chronic phase CML patients<sup>39</sup>, they do not efficiently kill leukemia stem cells (LSCs)<sup>62</sup>. It is generally accepted that eradication of LSCs is required for curing CML. On the one hand, LSCs share similar mechanisms for self-renewal and survival with normal hematopoietic stem cells (HSCs). For example, some developmental genes essential for normal HSCs, including *Wnt*, *Hedgehog*, *Bmi-1*, and *p53*, are involved in the regulation of both LSCs and HSCs<sup>89-92</sup>. On the other hand, LSCs utilize pathways relatively specific for LSCs<sup>85</sup>, providing a novel strategy for targeting LSCs while sparing normal HSCs. The challenge lies in the identification of genes that play a critical role in survival regulation of LSCs.

Mammalian bone marrow provides a relatively hypoxic niche essential for maintaining the self-renewal and survival of primitive HSCs<sup>175,176</sup>. It is widely accepted that gradients of O<sub>2</sub> from below 1% in the hypoxic niche to 6% in the sinusoidal cavity exist in human bone marrow<sup>175</sup>. The hypoxic niche is crucial for bone marrow function because *in vitro* culture of hematopoietic progenitors under hypoxic conditions displays a decrease in cellular proliferation accompanied by accumulation of the cells at the G0 phase of the cell cycle<sup>177,178</sup>. Although the molecular mechanisms by which hypoxia



regulates HSCs remains largely unknown, accumulating evidence suggests that hypoxia-inducible factor 1 (HIF1) plays a critical role in mediating the effect of hypoxia on HSCs<sup>177,179,180</sup>. In addition, it has been shown that cyclin-dependent kinase inhibitors that are regulated by HIF1 $\alpha$  are associated with the function of hypoxia in HSCs<sup>180</sup>. A recent study on HSCs in an animal model showed that regulation of the HIF1 $\alpha$  level is essential for the survival of HSCs<sup>181</sup>. In this study, it was found that normal HSCs maintain intracellular hypoxia and stabilize HIF1 $\alpha$  protein, and *HIF1 $\alpha$*  deficiency resulted in the loss of cell cycle quiescence in a p16<sup>Ink4a</sup>/p19<sup>Arf</sup> dependent manner, and subsequently impaired the reconstitution ability of HSCs during various stress settings including serial transplantation, myelosuppression and aging.

HIF1 belongs to the family of basic helix-loop-helix (bHLH) transcription factors, and is a heterodimer that consists of a constitutively expressed HIF1 $\beta$  subunit and a HIF1 $\alpha$  subunit<sup>122</sup>. The expression of HIF1 $\alpha$  is regulated in multiple ways. Under normoxic conditions, HIF1 $\alpha$  is degraded rapidly, which is triggered by the oxygen-hydroxylation of proline residues 402 and 564 by a proline hydroxylase (PHD). The hydroxylated HIF1 $\alpha$  is recognized by the Von Hippel-Lindau protein (pVHL), which is the recognition component of an E3 ubiquitin-protein ligase<sup>123,124</sup>. HIF1 $\alpha$  expression is also regulated by growth factors, cytokines and other signaling pathways such as PI3K and MAPK pathways<sup>125</sup>.

Recent studies demonstrate that HIF1 $\alpha$  plays a role in cancer progression by activating transcriptional programs for maintaining the ability of self-renewal and multipotency of cancer stem cells in a hypoxic environment<sup>126-128</sup>, and that HIF1 $\alpha$  was

required for stem cell functions of mouse lymphoma and human acute myeloid leukemia<sup>182</sup>. However, it is still unknown whether HIF1 $\alpha$  plays a role in LSCs in CML. In this study we show that *HIF1 $\alpha$*  is up-regulated in BCR-ABL-expressing LSCs and is required for survival maintenance of LSCs in CML.

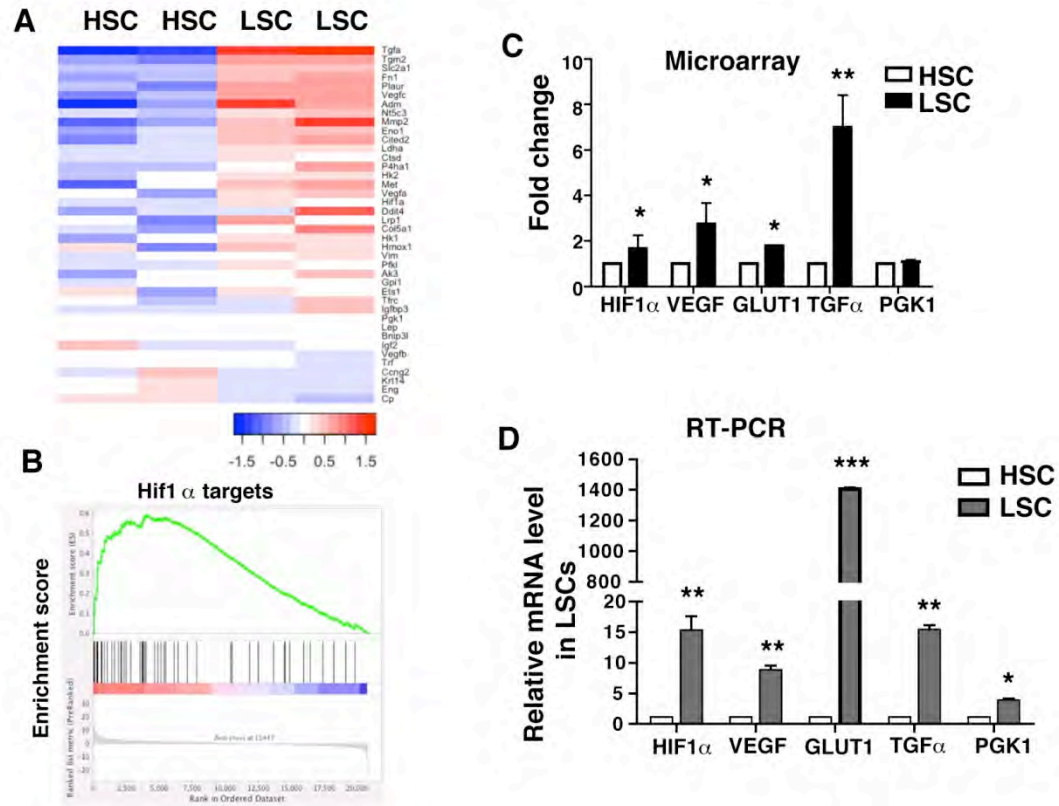
## Results

### Hypoxia-responsive genes are up-regulated by BCR-ABL in LSCs

We have shown that BCR-ABL-expressing HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells, LSK cells) function as LSCs in CML mice, because this cell population isolated from primary CML mice effectively transfers the disease into lethally irradiated secondary recipient mice<sup>63</sup>. This provides us with an assay system to examine whether BCR-ABL alters expression of hypoxia-responsive genes. We conducted a DNA microarray analysis to compare the gene expression profiles between LSCs and normal HSCs in our bone marrow transplantation (BMT) mouse model of CML (GEO submission: GSE10912). We retrovirally transduced bone marrow cells from C57BL/6J (B6) mice with BCR-ABL-GFP or GFP alone (as a normal HSC control) and transplanted the transduced cells into lethally irradiate B6 recipient mice to induce CML. Two weeks after BMT, we sorted GFP<sup>+</sup>LSK cells from bone marrow of the mice, and total RNA was extracted from the sorted cells for the Affymetrix microarray analysis. We intended to determine the global status of HIF1 $\alpha$  activity in LSCs, and found that the HIF1 $\alpha$  and its targets were up-regulated by BCR-ABL in LSCs as compared to the LSK cells transduced with GFP alone (**Fig. 1A, B**), which is enriched for normal HSCs. We next examined expression of

genes known to be specifically regulated by *HIF1 $\alpha$* , and found that expression of *VEGF*, *GLUT1* and *TGF $\alpha$* , except for *PGK1*, were significantly higher in LSCs than in normal HSCs (**Fig. 1C**). Real time RT-PCR assay confirmed the up-regulation of *HIF1 $\alpha$*  and hypoxia-responsive genes by BCR-ABL in LSCs (**Fig. 1D**). *PGK1* was also significantly up-regulated in LSCs (**Fig. 1D**). Together, these results suggest that HIF1 $\alpha$  is involved in functional regulation of LSCs and CML development.

**Figure 1. Hypoxia-responsive genes are up-regulated in LSCs.** LSCs and control HSCs were sorted from mice receiving BCR-ABL or empty vector-transduced bone marrow cells for DNA microarray analysis. **(A)** The heatmap compares the activation of the *HIF1 $\alpha$*  signaling pathway in LSCs and control HSCs. **(B)** Gene set enrichment analysis (GSEA) displays the expression profiling of *HIF1 $\alpha$*  targets in LSCs ( $P < 0.001$ ). **(C)** Microarray data showed higher expression of *HIF1 $\alpha$* , vascular endothelial growth factor (*VEGF*), glucose transporter type 1 (*GLUT1*), and transforming growth factor alpha (*TGF $\alpha$* ) in LSCs comparing to normal HSCs. **(D)** Real time RT-PCR was performed with primers specific for *HIF1 $\alpha$* , *VEGF*, *GLUT1*, *TGF $\alpha$* , and *phosphoglycerate kinase 1* (*PGK1*). The results were normalized using actin as a control, and shown as mean $\pm$ s.d. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .



**Figure 1. Hypoxia-responsive genes are up-regulated in LSCs.**

### HIF1 $\alpha$ is essential for CML development

To determine the role of HIF1 $\alpha$  in BCR-ABL leukemogenesis, we asked whether HIF1 $\alpha$  is required for CML development. Because deletion of the *HIF1 $\alpha$*  gene is embryonic lethal<sup>183</sup>, we crossed mice carrying a *loxP*-flanked *HIF1 $\alpha$*  allele with *Cre* transgenic mice in which expression of *Cre* is driven by the *Vav* regulatory element (**Fig. 2A**). Since *Vav* is an adaptor protein expressed predominantly in hematopoietic cells including the HSC population<sup>184</sup>, the use of the *Vav* regulatory element causes the deletion of the *HIF1 $\alpha$*  gene mainly in the hematopoietic system, allowing *Vav-Cre-HIF1 $\alpha$ <sup>flx/flx</sup>* mice to survive after *HIF1 $\alpha$*  deletion. To examine whether HIF1 $\alpha$  was efficiently deleted in *Vav-Cre-HIF1 $\alpha$ <sup>flx/flx</sup>* mice, we sorted LSK cells from bone marrow and analyzed *HIF1 $\alpha$*  expression by RT-PCR. We did not detect *HIF1 $\alpha$*  expression in LSK cells of *Vav-Cre-HIF1 $\alpha$ <sup>flx/flx</sup>* mice as compared to *HIF1 $\alpha$ <sup>flx/flx</sup>* mice (**Fig. 2A**). For simplicity, *Vav-Cre-HIF1 $\alpha$ <sup>flx/flx</sup>* mice will be referred to as *HIF1 $\alpha$ <sup>-/-</sup>* mice and *HIF1 $\alpha$ <sup>flx/flx</sup>* mice as wild type (WT) mice.

We first studied the role of HIF1 $\alpha$  in CML development using *HIF1 $\alpha$ <sup>-/-</sup>* mice in our bone marrow transplantation (BMT) model. Bone marrow cells from 5-FU-treated wild type (WT) or *HIF1 $\alpha$ <sup>-/-</sup>* mice were transduced with empty vector (GFP alone) or BCR-ABL-GFP retrovirus, and then transplanted into lethally irradiated recipient mice. While recipients of empty vector transduced WT or *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells did not develop CML disease, recipients of BCR-ABL-transduced WT or *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells developed and succumbed to CML within 4 weeks after BMT; however, no significant difference in survival was observed in WT and *HIF1 $\alpha$ <sup>-/-</sup>* CML mice (**Fig. 2B**),

which correlated with similar percentages at day 14 post BMT (**Fig. 2C**) and total numbers of myeloid leukemia cells in peripheral blood (PB) of CML mice as analyzed at day 8 and 11 post BMT (**Fig. 2D**). However, we noticed that at day 14 post BMT, the number of PB leukemia cells in mice receiving the BCR-ABL transduced *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells was significantly less than that in mice receiving the BCR-ABL transduced WT marrow cells (**Fig. 2D**). This observation suggests a possibility that *HIF1 $\alpha$*  plays a role in functional regulation of LSCs in CML. We also examined the spleens and bone marrow of CML mice, and found both WT and *HIF1 $\alpha$ <sup>-/-</sup>* CML mice displayed similar percentages of leukemia cells in the spleens and bone marrow at 2 weeks after BMT (**Fig. S1A**). Additionally, there was no significant difference in the degree of splenomegaly between the two groups of the CML mice (**Fig. 2E**).

Therefore, we decided to do a secondary transplantation assay to biologically assess the effect of *HIF1 $\alpha$*  on LSCs. We induced primary CML by transducing bone marrow cells from WT or *HIF1 $\alpha$ <sup>-/-</sup>* mice, followed by transplantation of the transduced cells into WT recipient mice. We then conducted a secondary BMT by transferring bone marrow cells from primary CML mice at day 14 to secondary recipient mice. Prior to the transplantation, we analyzed and normalized LSCs (GFP<sup>+</sup>LSK) from bone marrow of primary CML mice receiving BCR-ABL transduced WT or *HIF1 $\alpha$ <sup>-/-</sup>* donor bone marrow cells to ensure that equal numbers of LSCs were injected for the two transplantation groups. For control, we also transplanted equal numbers of normal GFP<sup>+</sup>LSK cells from primary control mice receiving empty vector transduced WT or *HIF1 $\alpha$ <sup>-/-</sup>* donor bone marrow cells into secondary recipient mice; to match up with the time frames for CML

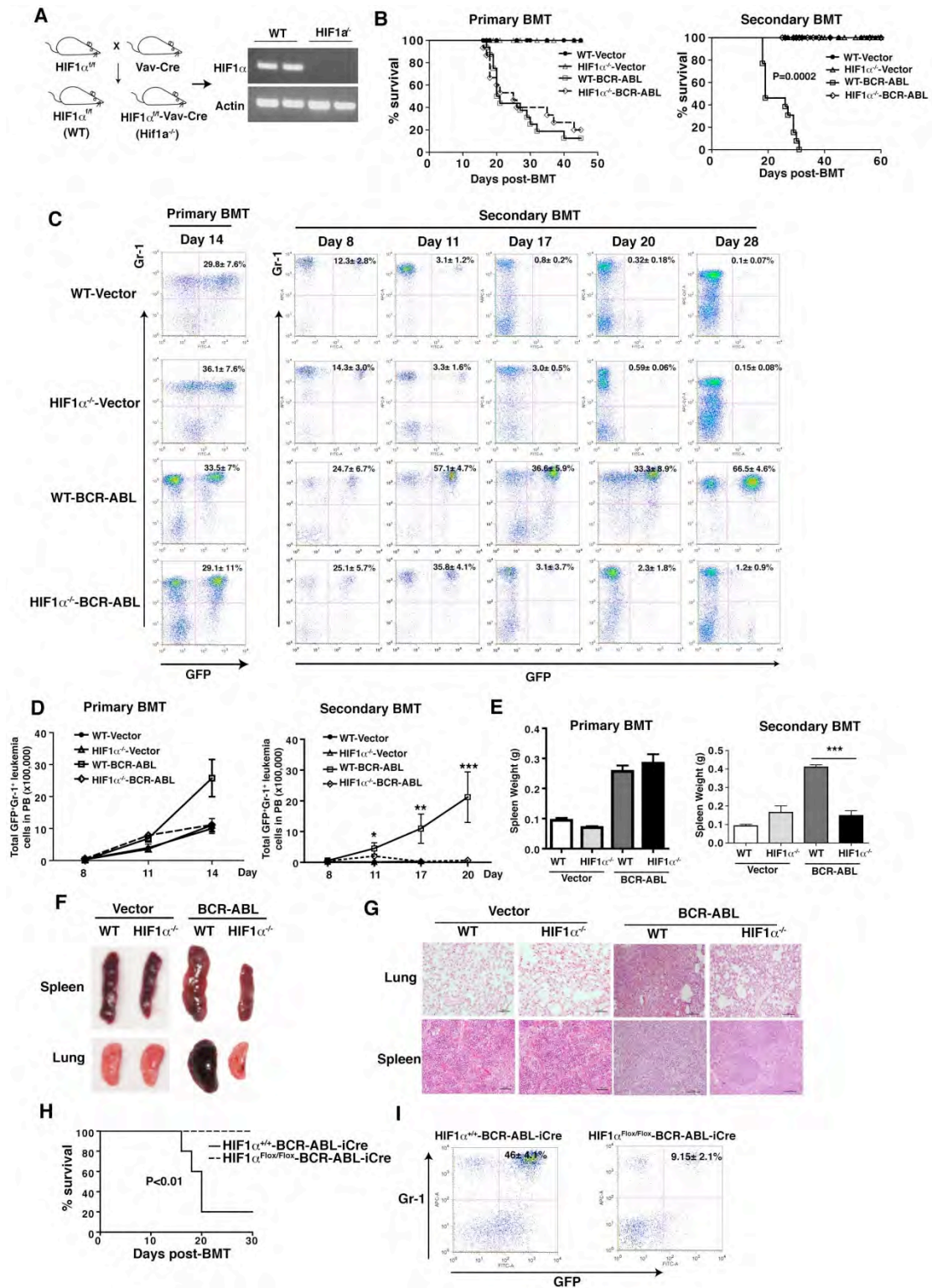
transplantation, we did secondary transplantation on day 14 after primary transplantation and analyzed the secondary recipient mice within a month. *HIF1 $\alpha$ <sup>-/-</sup>* LSCs failed to induce CML in the secondary recipient mice, whereas WT LSCs efficiently induced CML and all secondary recipient mice died within 4 weeks post BMT (**Fig. 2B**). The defective CML phenotype in the absence of *HIF1 $\alpha$*  was consistent with a gradual decrease of the percentages and total numbers of leukemia cells in peripheral blood (**Fig. 2C, D**). Unlike CML secondary transplantation (**Fig. 2C**), we observed that the percentages and total numbers of GFP<sup>+</sup> cells in peripheral blood of non-BCR-ABL mice gradually decreased to low levels in secondary recipients of WT or *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells (**Fig. 2C, D**), presumably because serial BMT within a short time period significantly increases the replicative stress, affecting the cycling ability of control HSCs<sup>185</sup>. Additionally, recipients of *HIF1 $\alpha$ <sup>-/-</sup>* LSCs displayed much less severe splenomegaly than did recipients of WT LSCs (**Fig. 2E, F**). Consistently, histological analysis showed no or modest infiltration of leukemia cells in the lung and spleen of the secondary recipient mice receiving *HIF1 $\alpha$ <sup>-/-</sup>* LSCs from primary CML mice (**Fig. 2G**). These results indicate that HIF1 $\alpha$  is required for CML development.

Constitutive expression of Cre may be toxic<sup>186</sup>. It is possible that Vav-driven expression of Cre causes damage on HSCs prior to BCR-ABL transduction, resulting in the defective CML development. In addition, 5FU is more detrimental to *HIF1 $\alpha$ <sup>-/-</sup>* HSCs<sup>181</sup>, potentially causing a defect in CML development. To examine these possibilities, we constructed BCR-ABL-iCre-GFP retroviral construct to allow co-expression of BCR-ABL and iCre in the same cell<sup>187</sup>. We transduced bone marrow cells from both WT and



*HIF1 $\alpha$ <sup>flow/flow</sup>* mice with BCR-ABL-iCre-GFP retrovirus, and transplanted into recipient mice. If Cre and 5-FU are toxic to bone marrow cells, both WT and *HIF1 $\alpha$ <sup>flow/flow</sup>* cells should be equally affected, allowing us to assess the additional effect of *HIF1 $\alpha$*  deletion on CML development. Recipients of BCR-ABL-iCre transduced WT and *HIF1 $\alpha$ <sup>flow/flow</sup>* bone marrow cells similarly developed CML, consistent with the findings for primary CML using WT and *HIF1 $\alpha$ <sup>-/-</sup>* mice (**Fig. 2B**). The secondary BMT confirmed the failure of CML development after deletion of *HIF1 $\alpha$*  (**Fig. 2H, I**).

**Figure 2. HIF1 $\alpha$  is essential for CML development.** (A) Deletion of *HIF1 $\alpha$*  in hematopoietic cells. Mice carrying the floxed *HIF1 $\alpha$*  allele were crossed with *Vav-Cre* transgenic mice in which the Vav promoter drives the expression of the Cre recombinase. RT-PCR analysis showed that *HIF1 $\alpha$*  was undetectable in sorted HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) from *HIF1 $\alpha$ <sup>flox/flox</sup>-Vav-Cre* mice. (B) Kaplan-Meier survival curves for primary and secondary recipients of empty vector or *BCR-ABL*-transduced bone marrow cells from WT or *HIF1 $\alpha$ <sup>-/-</sup>* donor mice. For secondary bone marrow transplantation, bone marrow cells from primary control and CML recipient mice which received empty vector or *BCR-ABL*-transduced WT and *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells were analyzed by FACS, and bone marrow cells containing equal number of WT or *HIF1 $\alpha$ <sup>-/-</sup>* GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells along with 2x10<sup>5</sup> WT BM cells (CD45.1) were transplanted into each lethally irradiated secondary recipient mouse. (C) FACS analysis of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of primary and secondary recipients of empty vector or *BCR-ABL*-transduced bone marrow cells from WT or *HIF1 $\alpha$ <sup>-/-</sup>* donor mice (n=5). Mean values ( $\pm$  s.d.) are shown. (D) The total numbers of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of primary and secondary recipients of empty vector or *BCR-ABL*-transduced bone marrow cells from WT or *HIF1 $\alpha$ <sup>-/-</sup>* donor mice (n=5). Mean values ( $\pm$  s.d.) are shown. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001. (E) The spleen weight of primary and secondary recipients of empty vector or *BCR-ABL*-transduced bone marrow cells from WT or *HIF1 $\alpha$ <sup>-/-</sup>* donor mice (n=12). Mean values ( $\pm$  s.d.) are shown. \*\*\* P<0.001. (F) Gross appearance of the lungs and spleens showed severe lung hemorrhages and splenomegaly in secondary recipients of WT LSCs but not *HIF1 $\alpha$ <sup>-/-</sup>* LSCs at day 14 after BMT, compared to those from recipients of WT and *HIF1 $\alpha$ <sup>-/-</sup>* HSCs. (G) Hematoxylin/eosin staining of tissue sections from lung and spleen of secondary recipients. The scale bar represents 50  $\mu$ m. (H) Kaplan-Meier survival curves for the secondary bone marrow transplantation. Bone marrow cells from WT or *HIF1 $\alpha$ <sup>flox/flox</sup>* mice were transduced with BCR-ABL-iCre-GFP retrovirus, and transplanted into lethally irradiated recipient mice. CML cells without *HIF1 $\alpha$*  failed to induce CML disease in the secondary BMT. (I) The percentages of leukemia cells in peripheral blood at day 14 in WT and *HIF1 $\alpha$ <sup>-/-</sup>* secondary CML mice.

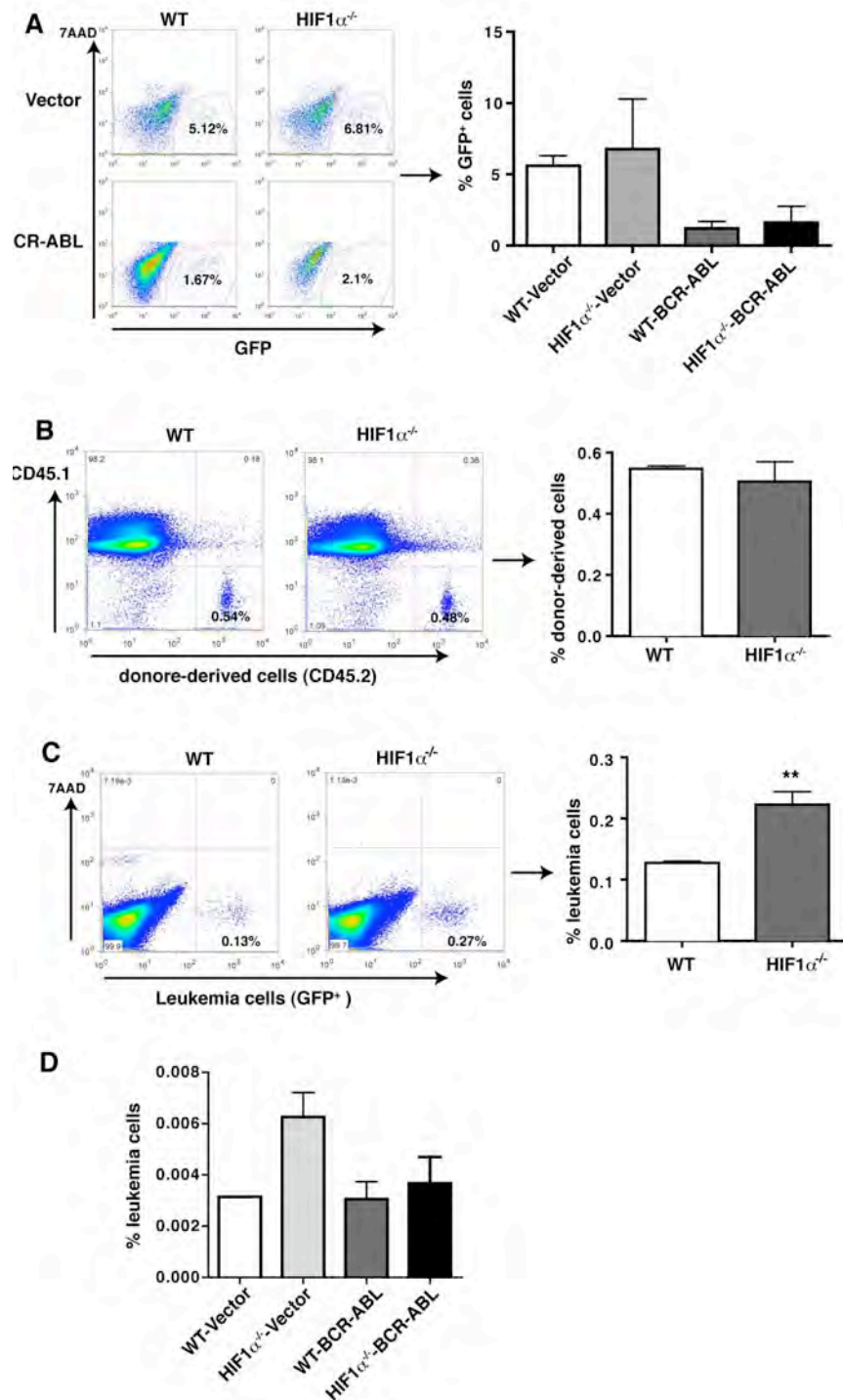


**Figure 2. HIF1α is essential for CML development.**

### **Deletion of HIF1 $\alpha$ does not affect transfection efficiency and the homing ability of leukemia cells**

The failure of CML induction in the secondary recipients could also be related to a change in retroviral transduction efficiency caused by the deletion of HIF1 $\alpha$ . To rule out this possibility, we compared GFP expression between empty GFP vector or BCR-ABL-GFP transduced WT and *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells by FACS prior to BMT, and found that the percentages of GFP<sup>+</sup> cells were similar between the transduced WT and *HIF1 $\alpha$ <sup>-/-</sup>* cells (**Fig. 3A**). These results indicate that HIF1 $\alpha$  deletion does not affect retroviral transduction efficiency. In addition, a failure of cell homing potentially resulted from the loss of HIF1 $\alpha$  may cause the failure of CML induction. To rule out this possibility, we performed an in vivo homing analysis comparing the ability of WT and *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells. Equal number of WT or *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells was injected into lethally irradiated CD45.1 mice. 3 hours after BMT, FACS analysis showed that the percentages of WT and *HIF1 $\alpha$ <sup>-/-</sup>* donor-derived cells representing by CD45.2 were similar (**Fig. 3B**). To confirm this result in BCR-ABL-expressing leukemia cells, we transplanted equal number of leukemia cells from bone marrow of primary CML mice receiving BCR-ABL-transduced WT or *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells into recipients. 3 hours after BMT, we did not observe a defective homing ability of BCR-ABL transduced *HIF1 $\alpha$ <sup>-/-</sup>* cells (**Fig. 3C**). Furthermore, using the same strategy, we compared the homing ability of WT or *HIF1 $\alpha$ <sup>-/-</sup>* LSCs with that of WT or *HIF1 $\alpha$ <sup>-/-</sup>* normal stem cells, and did not observe a reduced homing ability of *HIF1 $\alpha$ <sup>-/-</sup>* LSCs and normal stem cells (**Fig. 3D**).

**Figure 3. Effects of HIF1 $\alpha$  deletion on retroviral transduction efficiency and the homing ability of normal and BCR-ABL transduced total bone marrow cells or LSK cells.** (A) HIF1 $\alpha$  deletion does not affect retroviral transduction efficiency. WT and HIF1 $\alpha^{-/-}$  bone marrow cells were transduced with empty vector or BCR-ABL-GFP retrovirus, and 2 days later, the percentages of GFP $^{+}$  cells were determined by FACS. (B) FACS analysis showed the similar homing ability of WT and HIF1 $\alpha^{-/-}$  bone marrow cells.  $3 \times 10^6$  bone marrow cells from WT or HIF1 $\alpha^{-/-}$  mice (CD45.2) were transplanted into lethally irradiated recipients (CD45.1). The donor-derived bone marrow cells (CD45.2) were detected by FACS in 3 hours after BMT (n=4). Mean values ( $\pm$  s.d.) are shown. NS, no significance. (C) HIF1 $\alpha$  deletion does not cause a reduction of the homing ability of BCR-ABL transduced bone marrow cells.  $2 \times 10^6$  leukemia cells (GFP $^{+}$ ) from CML mice transplanted with BCR-ABL-transduced WT or HIF1 $\alpha^{-/-}$  bone marrow cells were transplanted into lethally irradiated recipients (CD45.1). 3 hours later, donor-derived leukemia cells (GFP $^{+}$ ) in the bone marrow were detected by FACS (n=4 for each group). Mean values ( $\pm$  s.d.) are shown. \*\*P<0.01. (D) HIF1 $\alpha$  deletion does not cause a reduction of the homing ability of control or BCR-ABL transduced stem cells. Bone marrow cells were transduced with GFP vector or BCR-ABL-GFP, and  $2 \times 10^4$  sorted normal HSCs and LSCs (GFP $^{+}$ Lin $^{-}$ Sca-1 $^{+}$ c-Kit $^{+}$ ) were transplanted into lethally irradiated recipients (CD45.1). 3 hours later, donor-derived leukemia cells (GFP $^{+}$ ) in the bone marrow were detected by FACS (n=3 for each group). Mean values ( $\pm$  s.d.) are shown.



**Figure 3. Effects of HIF1 $\alpha$  deletion on retroviral transduction efficiency and the homing ability of normal and BCR-ABL transduced total bone marrow cells or LSK cells.**

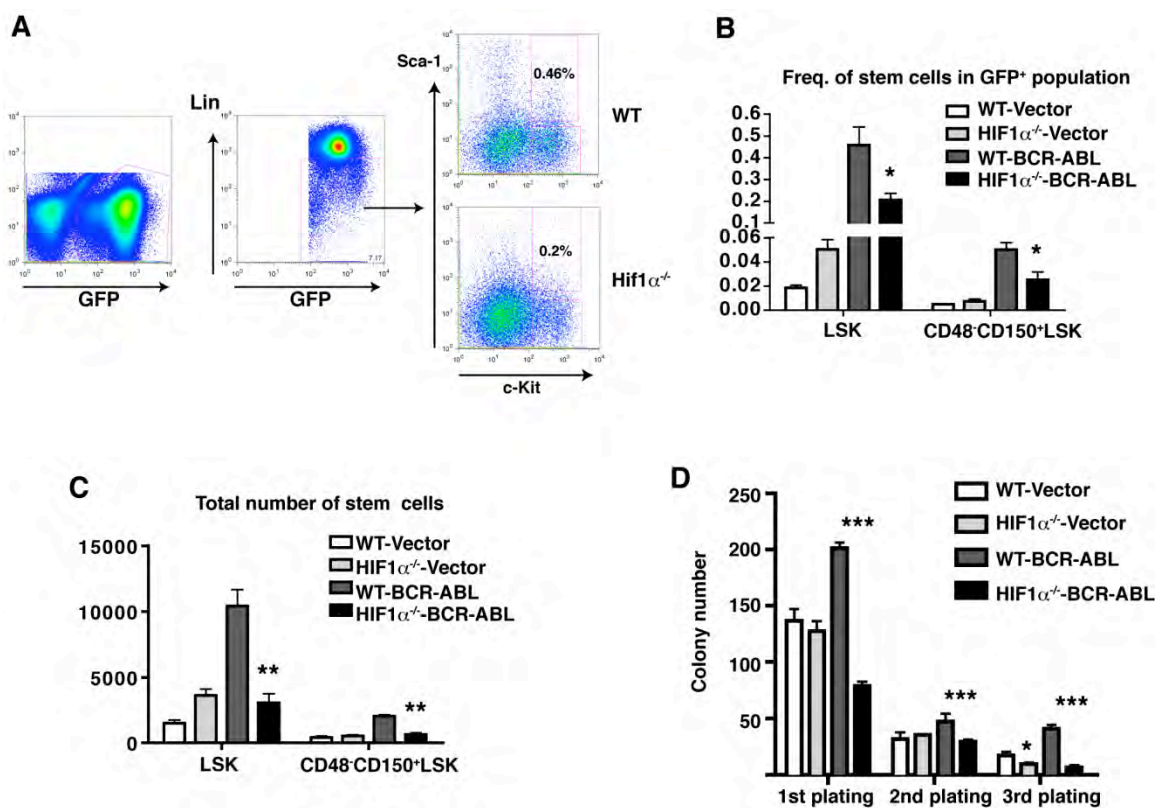
### **HIF1 $\alpha$ is essential for survival maintenance of LSCs**

Because CML is derived from a stem cell <sup>2</sup>, an inhibitory effect of HIF1 $\alpha$  deficiency on LSCs is likely a cause for the defect of CML development (Figure 2). To test this idea, we first examined LSCs in primary CML mice, and did not observe significant differences in the percentages and total numbers of LSCs (GFP<sup>+</sup>LSK and GFP<sup>+</sup>CD48<sup>-</sup>CD150<sup>+</sup>LSK) between WT and *HIF1 $\alpha$ <sup>-/-</sup>* CML mice (**Fig. S1B, C**), consistent with the similar survival of WT and *HIF1 $\alpha$ <sup>-/-</sup>* primary CML mice (**Fig. 2B**). We further analyzed LSCs in the secondary recipient mice receiving WT or *HIF1 $\alpha$ <sup>-/-</sup>* LSCs from primary CML mice (**Fig. 4A**). Two weeks post the secondary BMT, the percentages and numbers of LSCs (GFP<sup>+</sup>LSK and GFP<sup>+</sup>CD48<sup>-</sup>CD150<sup>+</sup>LSK) in the bone marrow were dramatically lower in the absence of HIF1 $\alpha$  (**Fig. 4B, C**), indicating that HIF1 $\alpha$  is required for survival maintenance of LSCs.

To investigate whether HIF1 $\alpha$  affects self-renewal of LSCs, we carried out an *in vitro* serial colony-forming assay. We sorted normal stem cells and LSCs (GFP<sup>+</sup>LSK) from control or CML mice receiving empty vector or *BCR-ABL*-transduced WT or *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells, and plated the cells *in vitro*. We observed that comparing to WT LSK cells, *HIF1 $\alpha$ <sup>-/-</sup>* LSK cells generated similar colonies in the 1<sup>st</sup> and 2<sup>nd</sup> plating, and less colonies in the 3<sup>rd</sup> plating. In contrast, comparing to WT LSCs, *HIF1 $\alpha$ <sup>-/-</sup>* LSCs gave rise to less colonies in all three plating, indicating that deletion of HIF1 $\alpha$  results in a more significant reduction of self-renewal ability of LSCs (**Fig. 4D**).

**Figure 4. HIF1 $\alpha$  deletion suppresses LSCs.** (A) FACS analysis of LSCs in bone marrow of secondary recipients of WT or HIF1 $\alpha$ <sup>-/-</sup> bone marrow cells from primary CML mice. (B, C) The percentages and total numbers of stem cells (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells and GFP<sup>+</sup>CD48<sup>-</sup>CD150<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells) in bone marrow of secondary recipient mice receiving bone marrow cells from WT or *HIF1 $\alpha$* <sup>-/-</sup> control and CML mice were analyzed by FACS. \* P<0.05; \*\* P<0.01. (D) The loss of *HIF1 $\alpha$*  causes a decrease in the colony-forming ability of LSCs. Sorted normal HSCs and LSCs (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells) were plated into methycellulose medium, colonies were counted and cells were serially replated. Data showed results from representative experiments. Mean values ( $\pm$  s.e.m) are shown. \*P<0.05; \*\*\*P<0.001.





**Figure 4. HIF1 $\alpha$  deletion suppresses LSCs.**

### **HIF1 $\alpha$ is required for survival maintenance of LSCs**

To understand the mechanism by which HIF1 $\alpha$  maintains the survival of LSCs, we analyzed the effect of HIF1 $\alpha$  on cell cycle progression and apoptosis of LSCs. We found that deletion of HIF1 $\alpha$  resulted in an accumulation of cells in the G0-G1 phase and a concomitant reduction in the S phase of the cell cycle (**Fig. 5A**), suggesting that HIF1 $\alpha$  deficiency impaired cell cycle progression of LSCs. HIF1 $\alpha$  is important for maintaining the quiescence of HSCs, and loss of HIF1 $\alpha$  promotes cell cycle entry<sup>180,181</sup>. Therefore, we further examined the cell cycle kinetics of *HIF1 $\alpha$ <sup>-/-</sup>* LSCs using Ki67. A reduction in the Ki67<sup>-</sup> G0 fraction and an increase in the Ki67<sup>+</sup> G1 fraction were observed in *HIF1 $\alpha$ <sup>-/-</sup>* LSCs, as compared to WT LSCs (**Fig. 5B**). These results indicate that HIF1 $\alpha$  regulates cell cycle progression of LSCs. Furthermore, by staining the cells with annexin V and 7AAD, we found that *HIF1 $\alpha$ <sup>-/-</sup>* LSCs had a higher apoptotic rate than WT LSCs (**Fig. 5D**). To further demonstrate the induction of apoptosis of LSCs by HIF1 $\alpha$  deficiency, we examined active caspase 3 in WT and *HIF1 $\alpha$ <sup>-/-</sup>* leukemia progenitors. We observed an increase in the number of active caspase 3-positive cells among *HIF1 $\alpha$ <sup>-/-</sup>* leukemia progenitor cells, as compared to WT cells (**Fig. 5E**). We also examined the effect of HIF1 $\alpha$  on cell cycle and apoptosis of normal HSCs, and unlike the observed effects on cell cycle progression and apoptosis of LSCs (**Fig. 5A, D; top panels**), we did not observe any significant differences between WT HSCs and *HIF1 $\alpha$ <sup>-/-</sup>* HSCs (**Fig. 5A, D; bottom panels**).

To understand the molecular mechanism by which HIF1 $\alpha$  regulates the function of LSCs, we compared the gene expression profiling between *HIF1 $\alpha$ <sup>-/-</sup>* LSCs and WT

LSCs (**Table 1 and 2**) and found that the cyclin-dependent kinase inhibitors  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  were up-regulated in  $HIF1\alpha^{-/-}$  LSCs. Real-time PCR confirmed higher levels of expression of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  in  $HIF1\alpha^{-/-}$  LSCs (**Fig. 5C**). Expression of another cyclin-dependent kinase inhibitor  $p57$  was also higher in  $HIF1\alpha^{-/-}$  LSCs than in WT LSCs (**Fig. 5C**). Elevated expression of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  was also observed in  $HIF1\alpha^{-/-}$  normal HSCs (**Fig. 5C**), which is consistent with previous studies<sup>181</sup>. However, expression of  $p57$  was unchanged in  $HIF1\alpha^{-/-}$  normal HSCs (**Fig. 5C**), indicating that loss of HIF1 $\alpha$  might not alter an identical set of genes in LSCs and normal HSCs. To test whether  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  mediate the function of HIF1 $\alpha$  in LSCs, we knocked down the expression of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  in LSCs using lentivirus-mediated shRNA to evaluate whether repression of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  rescues the defective LSCs function caused by HIF1 $\alpha$  deletion. Because the transcripts of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  share the exons 2 and 3, we designed a single shRNA to simultaneously knockdown expression of both genes in  $p53^{-/-}$  MEF cells that express higher levels of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  (**Fig. 5G**). In a colony-forming assay, we infected WT or  $HIF1\alpha^{-/-}$  CML bone marrow cells with the  $p16^{\text{Ink4a}}/p19^{\text{Arf}}$  shRNA, and found that  $HIF1\alpha^{-/-}$  CML bone marrow cells formed less numbers of colonies than did WT cells, and knockdown of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$  allowed the formation of more colonies in secondary and third replating. This result indicates that reduced function of  $HIF1\alpha^{-/-}$  LSCs was restored by repressing expression of  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$ , demonstrating that the regulatory function of HIF1 $\alpha$  in LSCs is at least in part mediated by  $p16^{\text{Ink4a}}$  and  $p19^{\text{Arf}}$ .

Because elevated p16<sup>Ink4a</sup> and p19<sup>Arf</sup> are related to the induction of stem cell senescence<sup>188,189</sup>, we examined the senescent status of *HIF1α*<sup>-/-</sup> LSCs and normal HSCs. Sorted LSCs and normal HSCs from WT and *HIF1α*<sup>-/-</sup> CML mice and control mice were stained with β-galactosidase. We observed a similar frequency of β-galactosidase-positive LSCs and normal HSCs (**Fig. 5H**), suggesting that senescence is not responsible for the impaired maintenance of *HIF1α*<sup>-/-</sup> LSCs. In addition, we also observed an increased expression of the apoptotic gene p53 in *HIF1α*<sup>-/-</sup> LSCs (**Fig. 5F**), which supports our observation of increased apoptosis of *HIF1α*<sup>-/-</sup> LSCs (**Fig. 5D**).

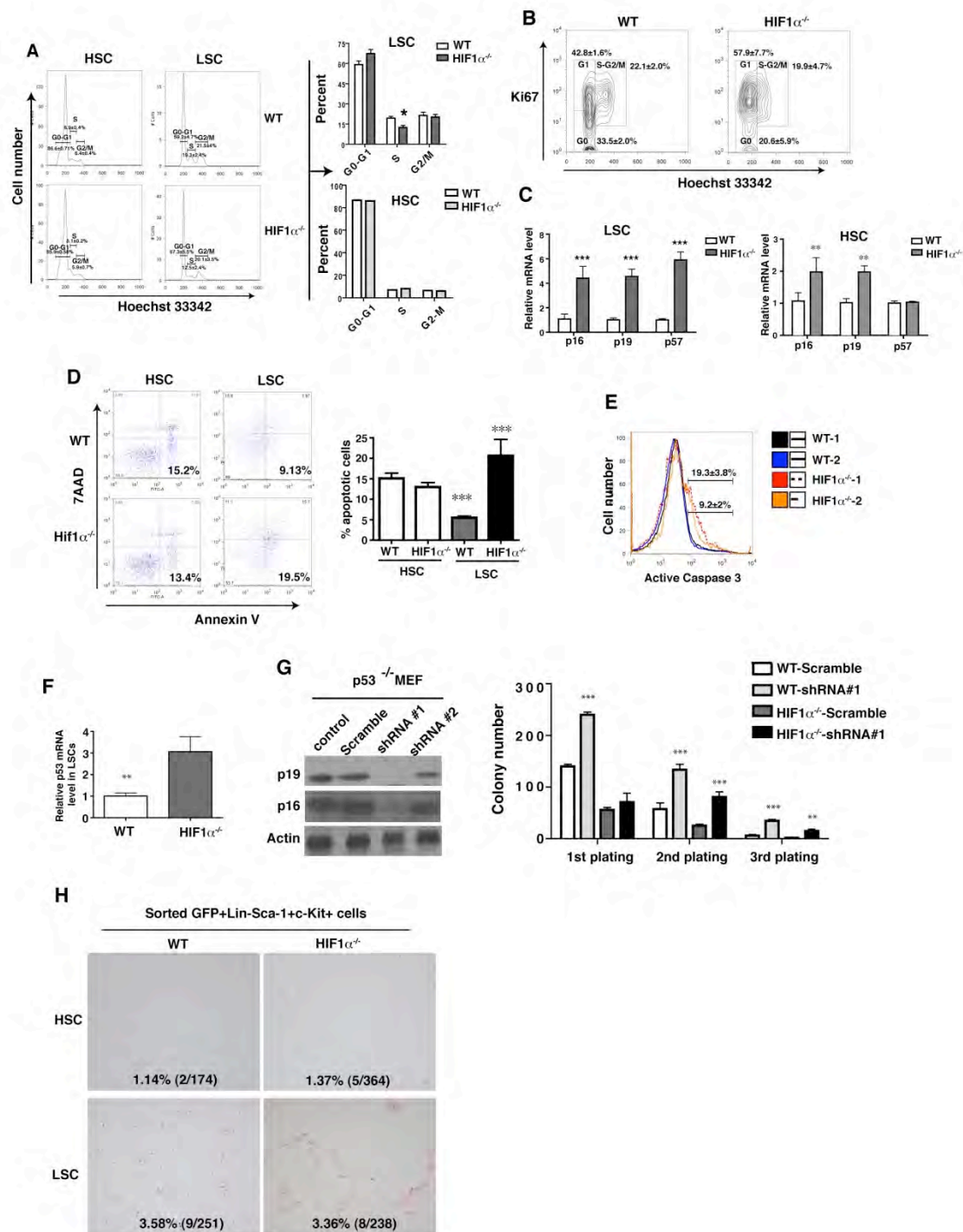
Table 1. List of up-regulated genes (*HIF1 $\alpha$* <sup>-/-</sup> LSCs vs WT LSCs)

Symbol	Gene name	Relative fold change, Log2
<i>Ces2g</i>	Carboxylesterase 2G	0.85
<i>Rhag</i>	Rhesus blood group-associated A glycoprotein	0.83
<i>Cda</i>	cytidine deaminase	0.81
<i>Cxcl10</i>	Chemokine (C-X-C motif) ligand 10	0.80
<i>Pklr</i>	Pyruvate kinase liver and red blood cell	0.78
<i>Crip2</i>	Cysteine-rich protein 2	0.71
<i>Epor</i>	Erythropoietin receptor	0.69
<i>Mmp14</i>	Matrix metalloproteinase 14 (membrane-inserted)	0.68
<i>Cd59a</i>	CD59a antigen	0.63
<i>Ccl19</i>	Chemokine (C-C motif) ligand 19	0.62
<i>Aco2</i>	Acyl-CoA thioesterase 2	0.59
<i>Car1</i>	Carbonic anhydrase 1	0.57
<i>Slc38a5</i>	Solute carrier family 38, member 5	0.57
<i>Bl2a1a</i>	B-cell leukemia/lymphoma 2-related protein A1a	0.55
<i>Ott</i>	Ovary testis transcribed	0.51
<i>Gpr50</i>	G-protein-coupled receptor 50	0.50
<i>Cpox</i>	Coproporphyrinogen oxidase	0.49
<i>Sphk1</i>	Sphingosine kinase 1	0.47
<i>Glo1</i>	Glyoxalase 1	0.45
<i>Nipa1</i>	Nonimprinted in Prader-Willi/Angelman syndrome 1 homolog (human)	0.45
<i>Plek2</i>	Pleckstrin 2	0.44
<i>Camsap11</i>	Calmodulin-regulated spectrin-associated protein 1-like 1	0.44
<i>Clcn2</i>	Chloride channel 2	0.43
<i>Slamf1</i>	Signaling lymphocytic activation molecule family member 1	0.43
<i>Pl4</i>	Platelet factor 4	0.42
<i>Mir224</i>	microRNA 224	0.41
<i>Hyal5</i>	Hyaluronoglucosaminidase 5	0.41
<i>Fbxw5</i>	F-box and WD-40 domain protein 5	0.40
<i>Rdh9</i>	Retinol dehydrogenase 9	0.40
<i>Stp4</i>	Secreted frizzled-related protein 4	0.38
<i>Ifnb1</i>	Interferon beta 1, fibroblast	0.38
<i>Car2</i>	Carbonic anhydrase 2	0.38
<i>Ubx2a</i>	UBX domain protein 2A	0.38
<i>Tns1</i>	Tensin 1	0.38
<i>Cited4</i>	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	0.37
<i>Mt2</i>	Metallothionein 2	0.37
<i>Trfr2</i>	Transferrin receptor 2	0.37
<i>Bcl11a</i>	B-cell CLL/lymphoma 11A (zinc finger protein)	0.36
<i>Fat3</i>	FAT tumor suppressor homolog 3 (Drosophila)	0.36
<i>Abhd6</i>	Abhydrolase domain-containing 6	0.35
<i>Id1</i>	Isopentenyl-diphosphate delta isomerase	0.35
<i>Ephb1</i>	Eph receptor B1	0.34
<i>Slc19a2</i>	Solute carrier family 19 (thiamine transporter), member 2	0.34
<i>Pou4f1</i>	POU domain, class 4, transcription factor 1	0.34
<i>Ifnab</i>	Interferon alpha B	0.34
<i>Pam1</i>	Prostate androgen-regulated mucin-like protein 1	0.34
<i>Trim58</i>	Tripartite motif-containing 58	0.34
<i>Phb</i>	Prohibitin	0.34
<i>Tnfrsf4</i>	Tumor necrosis factor (ligand) superfamily, member 4	0.32
<i>Hspe1</i>	Heat shock protein 1 (chaperonin 10)	0.32
<i>Ufsp1</i>	UFM1-specific peptidase 1	0.32
<i>Wdr55</i>	WD repeat domain 55	0.32
<i>Cdkn2a</i>	Cyclin-dependent kinase inhibitor 2A	0.12
<i>Tip53</i>	Transformation-related protein 53	0.1

Table 2. List of down-regulated genes (*HIF1α*<sup>-/-</sup> LSCs vs WT LSCs)

Symbol	Gene name	Relative fold change, Log2
<i>Mcp2</i>	Mast cell protease 2	-1.63
<i>Tnfrsf9</i>	Tumor necrosis factor receptor superfamily, member 9	-1.61
<i>Mcp1</i>	Mast cell protease 1	-1.57
<i>Rps25</i>	Ribosomal protein S25	-1.43
<i>Mcp4</i>	Mast cell protease 4	-1.33
<i>Bnip3</i>	BCL2/adenovirus E1B-interacting protein 3	-1.28
<i>Cox7c</i>	Cytochrome c oxidase, subunit viic	-1.20
<i>Tph1</i>	Tryptophan hydroxylase 1	-1.16
<i>Gzmb</i>	Granzyme B	-1.01
<i>Bnip3</i>	BCL2/adenovirus E1B-interacting protein 3	-0.96
<i>Plod2</i>	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	-0.94
<i>Ccr2</i>	Chemokine (C-C motif) receptor 2	-0.87
<i>Slc16a3</i>	Solute carrier family 16 (monocarboxylic acid transporters), member 3	-0.87
<i>Lipo1</i>	Lipase, member O1	-0.87
<i>Oma1</i>	Chymase 1, mast cell	-0.86
<i>Dntt</i>	Deoxynucleotidyltransferase, terminal	-0.86
<i>Xcr1</i>	Chemokine (C motif) receptor 1	-0.84
<i>Hmgb1</i>	High mobility group box 1	-0.83
<i>Marcks</i>	Myristoylated alanine-rich protein kinase C substrate	-0.82
<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	-0.81
<i>Serpine1</i>	Serine (or cysteine) peptidase inhibitor, clade E, member 1	-0.79
<i>Gimap4</i>	GTPase, IMAP family member 4	-0.77
<i>Akr1c12</i>	Aldo-keto reductase family 1, member C12	-0.74
<i>Itgae</i>	Integrin alpha E, epithelial associated	-0.70
<i>Ebf1</i>	Early B-cell factor 1	-0.69
<i>Maob</i>	Monoamine oxidase B	-0.68
<i>Tlr3</i>	Toll-like receptor 3	-0.66
<i>Cd36</i>	CD36 antigen	-0.66
<i>Kmo</i>	Kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	-0.66
<i>Il13ra1</i>	Interleukin 13 receptor, alpha 1	-0.66
<i>Stat4</i>	Signal transducer and activator of transcription 4	-0.64
<i>Slamf7</i>	SLAM family member 7	-0.63
<i>Il7r</i>	Interleukin 7 receptor	-0.61
<i>Cdkn1b</i>	Cyclin-dependent kinase inhibitor 1B	-0.60
<i>Suonr1</i>	Succinate receptor 1	-0.60
<i>Irf8</i>	Interferon regulatory factor 8	-0.59
<i>Cd74</i>	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	-0.58
<i>Nov</i>	Nephroblastoma overexpressed gene	-0.56
<i>Ptprg</i>	Protein tyrosine phosphatase, receptor type, G	-0.55
<i>Socs2</i>	Suppressor of cytokine signaling 2	-0.53
<i>Irf5</i>	Interferon regulatory factor 5	-0.51
<i>Igfbp5</i>	Insulin-like growth factor-binding protein 5	-0.50
<i>Slamf6</i>	SLAM family member 6	-0.50
<i>Slc2a1</i>	Solute carrier family 2 (facilitated glucose transporter), member 1	-0.50
<i>Left</i>	Lymphoid enhancer-binding factor 1	-0.49
<i>Cd96</i>	CD96 antigen	-0.49

**Figure 5. HIF1 $\alpha$  is required for survival maintenance of LSCs.** (A) The cell cycle analysis of normal HSCs and LSCs. The percentages of *HIF1 $\alpha$* <sup>-/-</sup> LSCs were low in the S phase and higher in the G0-G1 phase, as compared to WT LSCs (n=6). However, normal WT and *HIF1 $\alpha$* <sup>-/-</sup> HSCs showed similar percentages of cells in G0-G1, S and G2-M phases. (B) The cell cycle analysis of LSCs using Ki67 and Hoechst 33342 showed an accumulation of G1 phase of *HIF1 $\alpha$* <sup>-/-</sup> LSCs. (C) RT-PCR analysis showed elevated expression of cell cycle inhibitors *p16*<sup>Ink4a</sup>, *p19*<sup>Arf</sup>, and *p57* in the sorted HSCs and LSCs from *HIF1 $\alpha$* <sup>-/-</sup> control and CML mice as compared to the sorted HSCs and LSCs from WT control and CML mice. Bone marrow cells from control and CML mice at day 14 post BMT were collected, and HSCs and LSCs were sorted for extracting total RNA. Mean values ( $\pm$  s.d.) are shown \*\* P<0.01; \*\*\* P<0.001. (D) The apoptosis of normal HSCs and LSCs from bone marrow of control and CML mice (n=6). HIF1 $\alpha$  deletion induced the apoptosis of LSCs, but did not affect the apoptosis of normal HSCs. (E) FACS analysis showed a higher percentage of active caspase positive *HIF1 $\alpha$* <sup>-/-</sup> leukemia progenitors. (F) RT-PCR analysis showed elevated expression of *p53* in the sorted LSCs from *HIF1 $\alpha$* <sup>-/-</sup> CML mice comparing to the sorted LSCs from control CML mice. Mean values ( $\pm$  s.d.) are shown. \*\* P<0.01. (G) Simultaneous knockdown of *p16*<sup>Ink4a</sup> and *p19*<sup>Arf</sup> rescued the colony-forming ability of *HIF1 $\alpha$* <sup>-/-</sup> LSCs. Western blot analysis showed that protein levels of *p16*<sup>Ink4a</sup> and *p19*<sup>Arf</sup> in *p53*<sup>-/-</sup> MEF and CML leukemia cells were dramatically reduced by shRNA#1 that targets both *p16*<sup>Ink4a</sup> and *p19*<sup>Arf</sup>. Bone marrow cells from WT and *HIF1 $\alpha$* <sup>-/-</sup> normal or CML mice were infected by shRNA#1, and plated into methycellulose media after selection by puromycin. Colonies were counted and cells from the colonies were serially replated. Mean values ( $\pm$  s.d.) are shown \*\* P<0.01; \*\*\* P<0.001. (H) Loss of HIF1 $\alpha$  did not affect the senescence of normal HSCs and LSCs. Sorted GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells from the bone marrow of WT or *HIF1 $\alpha$* <sup>-/-</sup> control and CML mice were stained by X-Gal, and the number of  $\beta$ -galactosidase expressing cells were counted.



**Figure 5. HIF1 $\alpha$  is required for survival maintenance of LSCs**



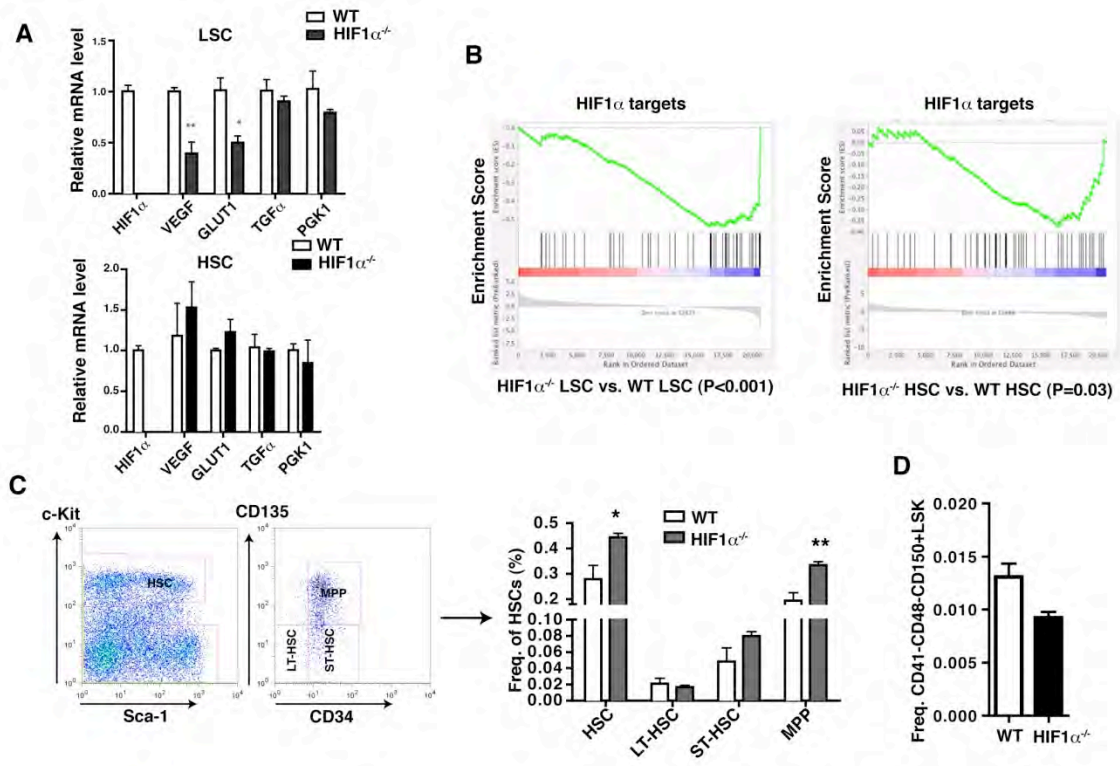
### Loss of HIF1 $\alpha$ has different effects on LSCs and normal HSCs

To further examine the involvement of HIF1 $\alpha$  in functional regulation of LSCs, we examined whether deletion of HIF1 $\alpha$  causes reduced expression of several *HIF1 $\alpha$*  target genes. We found that as compared to WT LSCs, expression levels of *VEGF* and *GLUT1* in *HIF1 $\alpha$ <sup>-/-</sup>* LSCs were reduced by approximately 50% (**Fig. 6A**); however, there were no significant differences in the expression of *TGF  $\alpha$*  and *PGK1* between *HIF1 $\alpha$ <sup>-/-</sup>* and WT LSCs (**Fig. 6A**). These results suggest that these hypoxia-responsive genes are responsive to *HIF1 $\alpha$*  in LSCs. To determine whether loss of HIF1 $\alpha$  also similarly affects normal HSCs, we sorted HSCs from bone marrow of WT and *HIF1 $\alpha$ <sup>-/-</sup>* bone marrow cells, and compared expression of these HIF1 $\alpha$  target genes by RT-PCR. We found that loss of HIF1 $\alpha$  did not cause a significant reduction of expression of *VEGF* and *GLUT1* in normal HSCs, although expression of these two genes was down-regulated in *HIF1 $\alpha$ <sup>-/-</sup>* LSCs (**Fig. 6A**). Furthermore, we conducted a microarray assay to examine expression of HIF1 $\alpha$  target genes in *HIF1 $\alpha$ <sup>-/-</sup>* LSCs and *HIF1 $\alpha$ <sup>-/-</sup>* HSCs and to evaluate the data using GSEA algorithm<sup>190,191</sup>. The enrichment plots showed that *HIF1 $\alpha$*  targets were generally down-regulated in both LSCs and *HIF1 $\alpha$ <sup>-/-</sup>* HSCs, but the extent of this downregulation was much more significant in *HIF1 $\alpha$ <sup>-/-</sup>* LSCs than *HIF1 $\alpha$ <sup>-/-</sup>* HSCs (P<0.001 vs. P<0.03) (**Fig. 6B**). Together, these results suggest that relative to normal HSCs, LSCs are more dependent on the HIF1 $\alpha$  pathway.

The differential expression of the HIF1 $\alpha$  target genes in LSCs and HSCs prompted us to further examine the biological effect of HIF1 $\alpha$  on normal HSCs in our *Vav-Cre-HIF1 $\alpha$ <sup>fllox/fllox</sup>* mice. It is also necessary to point out that in our *Vav-Cre-*

*HIF1 $\alpha$ <sup>flx/flx</sup>* mice, *HIF1 $\alpha$*  was deleted by the *Vav-Cre* in contrast to the deletion of *HIF1 $\alpha$*  by *Mx1-Cre*<sup>181</sup>. We observed that the percentage of LSK cells (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) in *HIF1 $\alpha$ <sup>-/-</sup>* mice was significantly higher than that in WT mice (**Fig. 6C**). The higher percentage of LSK cells in *HIF1 $\alpha$ <sup>-/-</sup>* mice was largely due to the effect of *HIF1 $\alpha$*  deletion on MPP population (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>Flt3<sup>+</sup>) and ST-HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>Flt3<sup>-</sup>), but not on LT-HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>-</sup>Flt3<sup>-</sup>), as the percentage of LT-HSCs was only slightly lower in *HIF1 $\alpha$ <sup>-/-</sup>* mice than in WT mice (**Fig. 6C**). Furthermore, a lower percentage of CD41<sup>-</sup>CD48<sup>-</sup>CD150<sup>+</sup>LSK was also found in *HIF1 $\alpha$ <sup>-/-</sup>* mice as compared to WT mice (**Fig. 6D**). This is in line with previous study showing that *HIF1 $\alpha$* -null HSC are still able to support hematopoiesis in physiological conditions<sup>181</sup>.

**Figure 6. Effects of HIF1 $\alpha$  deficiency on normal HSCs.** (A) RT-PCR analysis for the expression of *HIF1 $\alpha$* , *VEGF*, *GLUT1*, *TGF $\alpha$* , and *PGK1* in WT or *HIF1 $\alpha$ <sup>-/-</sup>* normal HSCs and LSCs. (B) Gene set enrichment analysis of DNA microarray data displays gene expression profiling of HIF1 $\alpha$  targets in WT or *HIF1 $\alpha$ <sup>-/-</sup>* normal HSCs and LSCs. (C) FACS analysis of HSCs in bone marrow cells from WT or *HIF1 $\alpha$ <sup>-/-</sup>* mice. The percentages of LSK cells (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>), LT-HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>-</sup>CD135<sup>-</sup>) and ST-HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>-</sup>CD135<sup>+</sup>) were similar in WT and *HIF1 $\alpha$ <sup>-/-</sup>* mice; however, *HIF1 $\alpha$ <sup>-/-</sup>* mice showed higher percentage of MPP (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>CD135<sup>+</sup>) (n=5). (D) Similar percentages of CD41<sup>-</sup>CD48<sup>-</sup>CD150<sup>+</sup> Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells in bone marrow cells of WT or *HIF1 $\alpha$ <sup>-/-</sup>* mice (n=3).



**Figure 6. Effects of *HIF1 $\alpha$*  deficiency on normal HSCs**

## Discussion

HIF has been shown to be involved in cancer development<sup>122,128,192</sup>. In this study, we utilized mice with conditional deletion for HIF1 $\alpha$  to provide the first evidence for an essential role of HIF1 $\alpha$  in CML development and maintenance of LSCs. Because HIF1 $\alpha$  deficiency results in elevated expression of cell cycle inhibitors p16<sup>Ink4a</sup>, p19<sup>Arf</sup> and p57 and apoptotic gene p53 in LSCs, inhibition of the HIF1 $\alpha$  pathway is likely to be effective in eradicating LSCs, providing a rationale for developing an anti-HIF1 $\alpha$  therapy in treating CML.

We demonstrate that HIF1 $\alpha$  is required for survival regulation of LSCs in CML mice, and that *HIF1 $\alpha$*  and its target genes are up-regulated in LSCs by BCR-ABL, which is consistent with BCR-ABL up-regulation of *HIF1 $\alpha$*  mRNA expression in a leukemia cell line<sup>129</sup>. The PI3K and mTOR pathways appear to be responsible for BCR-ABL-dependent *HIF1 $\alpha$*  expression in Ba/F3 cells<sup>129</sup>. Consistent with a previous report showing a higher level of VEGF expression in the plasma and serum of CML patients<sup>193</sup>, we show that expression of *VEGF* is elevated in LSCs in CML mice. We also show a higher level of *GLUT1* expression in LSCs, consistent with a previous observation that BCR-ABL expression alters the distribution of GLUT1 in cell plasma membrane of TonB210 cells<sup>194</sup>. Interestingly, HIF1 $\alpha$  deletion causes an increased homing ability of BCR-ABL-expressing leukemia cells to bone marrow, consistent with increased expression of some homing-related genes such as MMP-14, CXCL10, CCL19 (data not shown). The underlying mechanisms need to be further studied in the future.

Our study suggests that HIF1 $\alpha$  deletion induces a tumor suppressor response in

LSCs through induction of expression of p16<sup>Ink4a</sup>, p19<sup>Arf</sup>, and p53. In addition, the elevated expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> are not specific to LSCs, as HIF1 $\alpha$  deficiency also induces their expression in normal HSCs<sup>181</sup>. This is consistent with a previous study showing that the enforced expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> in HSCs resulted in cell cycle arrest and p53-dependent apoptosis, and caused the exhaustion of HSCs<sup>90</sup>. Our data suggest that p16<sup>Ink4a</sup> and p19<sup>Arf</sup> contribute to the exhaustion of LSCs. On the other hand, it is known that HIF1 $\alpha$  is essential for cell cycle arrest during hypoxia<sup>195</sup>. HSCs cultured in hypoxia *in vitro* increased their long-term reconstitution ability through maintaining quiescence of HSCs<sup>180</sup>. Because HIF1 $\alpha$  deletion induces the loss of LSC quiescence and accumulation of the cells in the G1 phase, up-regulated expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> induced by HIF1 $\alpha$  deletion may inhibit the transition of G1/S phase of cell cycle through binding to cyclin D-dependent CDK4/6<sup>196</sup>. Indeed, we observed an increase of *HIF1 $\alpha$* <sup>-/-</sup> LSCs in G1 phase and a decrease of the cells in S phase. Furthermore, knockdown of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> rescues the defect of *HIF1 $\alpha$* <sup>-/-</sup> LSCs to form colonies. It has been reported that *Ink4a-Arf*<sup>-/-</sup> HSCs retain their self-renewal capacity better than wild type HSCs in a long-term ex vivo culture<sup>197</sup>, and repression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> by overexpressing Bmi1 restores the reconstitution ability of *HIF1 $\alpha$* <sup>-/-</sup> HSCs<sup>181</sup>. However, other cell cycle regulators might also play a role in mediating the function of HIF1 $\alpha$  in LSCs, because up-regulated expression of p21 and p27 in *HIF1 $\alpha$* -deficient cells is also associated with an increased number of cells in the G1 phase and a decreased number of cells in the S phase of the cell cycle<sup>126</sup>. Mechanistically, it is also possible that cellular senescence induced by HIF1 $\alpha$  deficiency facilitates the exhaustion of *HIF1 $\alpha$* <sup>-/-</sup> LSCs and

HSCs, which is likely caused by the upregulation of p16<sup>ink4a</sup> and p19<sup>Arf</sup>. Although a previous work suggests that HIF1 $\alpha$  plays a protective role against senescence<sup>181</sup>, a solid supporting evidence is still lacking. In fact, we observe that HIF1 $\alpha$  deficiency causes decreased proliferation of LSCs, indicating a different mechanism.

HIF1 $\alpha$  appears to play a protective role under stress setting, such as in vitro culture, 5-FU treatment and oncogene transformation. In our study, we use the Vav promoter to drive Cre expression in order to delete the *HIF1 $\alpha$*  gene predominantly in hematopoietic system. Another advantage of using Vav-driven Cre expression is to avoid the effect of IFN signaling on LSCs and HSCs in the popularly used Mx1-Cre expression system<sup>198,199</sup>. Furthermore, *Vav-Cre-HIF1 $\alpha$ <sup>flox/flox</sup>* mice are viable and has a basically normal lifespan. Thus, our *Vav-Cre-HIF1 $\alpha$ <sup>flox/flox</sup>* mice will provide a valuable model for in-depth study of the role of HIF1 $\alpha$  in LSCs and BCR-ABL leukemogenesis for developing novel therapeutic strategies in CML treatment.

## Materials and Methods

**Mice.** *HIF1 $\alpha$ <sup>flox/flox</sup>* C57BL/6J, Vav1-Cre-C57BL/6J, C57BL/6J-CD45.1 and C57BL/6J-CD45.2 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). All mice were bred and maintained in a temperature- and humidity-controlled environment and given unrestricted access to 6% chow diet and acidified water.

**Generation of retroviral stocks.** The retroviral constructs MSCV-IRES-GFP, MSCV-BCR-ABL-IRES-GFP were used to generate high-titer, helper-free, replication-defective ecotropic viral stocks by transient transfection of 293T cells as previously described<sup>23</sup>.

The MSCV-BCR-ABL-iCre-GFP construct was made by cloning the iCre ORF into the MSCV-BCR-ABL vector, and cloning the IRES-GFP fragment after the iCre ORF, as described previously<sup>187</sup>.

**Bone marrow transduction/transplantation.** 8 to 12 week-old C57BL/6 mice were used for bone marrow transduction/transplantation. Retroviral transduction and transplantation of mouse bone marrow cells for inducing CML by BCR-ABL had been described previously<sup>23,86</sup>. For secondary transplantation, equal number of bone marrow cells from primary CML mice was transplanted into lethally irradiated recipient mice.

**Flow cytometry analysis.** Hematopoietic cells were collected from bone marrow and peripheral blood of CML mice for FACS analysis. For stem cell analysis, bone marrow cells were incubated with biotin-labeled lineage antibody cocktail containing a mixture of antibodies against CD3, CD4, CD8, B220, Gr-1, Mac-1 and Ter119. After washing, the fluorochrome-labeled secondary antibody (APC-Cy7-conjugated Streptavidin) for recognizing biotin and PE-conjugated c-Kit and APC-conjugated Sca-1 antibodies were added to the cells. Long-term and short-term stem cells were distinguished by CD34 and CD135 or SLAM markers CD41, CD48, and CD150. All these antibodies were purchased from eBioscience.

Cell cycle analysis of LSCs was performed by staining cells with antibodies in combination with Hoechst 33342, followed by flow cytometric analysis. For Ki67 staining, cells were fixed and permeabilized before staining with PerCP-Cy5.5-conjugated anti-Ki67 antibody (from BD Biosciences). To analyze apoptosis of bone marrow cells, the cells were stained with Annexin V (from eBioscience). 7AAD was



added before flow cytometry analysis. For active caspase 3 staining, cells were first surface stained, fixed and permeabilized (Fixation and Permeabilization kit; eBioscience), and stained with PE-conjugated anti-active caspase 3 antibody (from BD Biosciences).

***In vitro* methylcellulose colony formation assay.** Normal stem cells or LSCs (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) from bone marrow of control and CML mice receiving empty vector or BCR-ABL-transduced WT or *HIF1α*<sup>-/-</sup> bone marrow cells were sorted by FACS, and were cultured in methylcellulose medium (Methocult GF M3434; Stem Cell Technologies) for 7 days. Colonies were counted under microscope. For the colony forming assay after shRNA-mediated knockdown of CDKN2A, CML bone marrow cells were cultured in methylcellulose media containing 2.5μg/mL puromycin for selection.

**Senescence-associated β-galactosidase staining.** LSCs (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) from bone marrow of CML mice were sorted by FACS, and cytopun on a poly-lysine coated slide and stained using the senescence-associated β-gal staining kit (#9860, Cell Signaling Technology).

**Lentiviral transduction and shRNA-mediated knockdown of p16<sup>Ink4a</sup> and p19<sup>Arf</sup>.**

Lentiviral shRNA vector pLKO.1 were purchased from OpenBiosystems. Sequences of CDKN2a (p16<sup>Ink4a</sup> and p19<sup>Arf</sup>) shRNA were as follows: shRNA#1: sense 5'-gtgatgatgatgggcaacgtt-3': shRNA #1 antisense 5'-aacgttgcccatcatcatcac-3'; shRNA#2 sense 5'-gctcggctggatgtgcgcgat-3' #2 antisense 5'-atcgcgcacatccagccgagc-3'. For transduction of the CDKN2A shRNAs, p53<sup>-/-</sup>MEF or CML bone marrow cells were

transfected with lentivirus-shRNA, and 24 hours later, were selected under puromycin (2.5µg/ml) for 48 hours before the next steps.

**Antibodies and western blot analysis.** Antibodies against p16<sup>Ink4a</sup>, p19<sup>Arf</sup> and actin were purchased from Santa Cruz Biotechnology. Protein lysates were prepared by lysing cells in immunoprecipitation buffer, and Western blotting were carried out as shown previously<sup>200</sup>.

**Real Time-PCR.** Total RNA was isolated from GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> bone marrow cells from mice using the RNeasy Mini kit (Qiagen, CA). cDNA was synthesized using the Ovation-Pico cDNA synthesis method. All real time PCR reactions were done using the Applied Biosystems 7500. 25µL reaction system was composed of 12.5µL SYBR Green, 2.5µL 20uM primer mixture, 10ng cDNA and nuclease-free water. All experiments were performed in triplicate. Beta-actin was the internal control. The primer sequences are shown as following: *HIF1a* sense: tgagcttgctcatcagttgc; *HIF1a* antisense: ccatctgtgccttcattca; *VEGF* sense: caggctgctgaacgatgaa; *VEGF* antisense: tttcttgcgctttcggtttt; *GLUT1* sense: gctgtgcttatgggcttctc; *GLUT1* antisense: agaggccacaagtctgcatt; *TGFa* sense: agcatgtgtctgccactctg; *TGFa* antisense: tggatcagcacacaggtgat; *PGK1* sense: caaggctttggagagtccag; *PGK1* antisense: tgtgccaatctccatgtgt; *p16<sup>Ink4a</sup>* sense: cgaactcttcggctgtaccc; *p16<sup>Ink4a</sup>* antisense: cgaatctgcaccgtagttgagc; *p19<sup>Arf</sup>* sense: gttcttggtcactgtgaggattcag; *p19<sup>Arf</sup>* antisense: ccatcatcatcacctggtccag; *p53* sense: agagaccgccgaacagaaga; *p53* antisense: ctgtagcatgggcatcctt; *p57* sense: ctgacctcagaccaattc; *p57* antisense: ctgagaccggctcagttc.

**DNA Microarray and Data Analysis.** Normal HSCs and LSCs (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>; 99% purity) were stored by FACS directly into RNAlater (Ambion, Austin, TX) and homogenized in RLT Buffer (RNeasy Micro Kit) (Qiagen, Valencia, CA). Total RNA was isolated by following the protocol for the RNeasy Micro Kit. RNA was amplified, labeled, and approximately 2.0µg of fragmented and biotin-labeled cDNA was then hybridized onto Mouse Genome 430 1.0 microarray (Affymetrix, Santa Clara, CA.). Gene expression values from Affymetrix cel files were background corrected and normalized using RMA algorithm, and were rank-ordered by the extent of differential expression using the moderated t-statistics from limma R package. To collectively assess HIF1α targets, the GSEA algorithm was used to evaluate the statistical significance of the expression of HIF1α targets<sup>122,190,191</sup>.

**Statistical analysis.** Results are given as mean ± s.d. Statistical analysis was performed by Students' t test. For survival curves, p values were obtained using a Log-rank test.

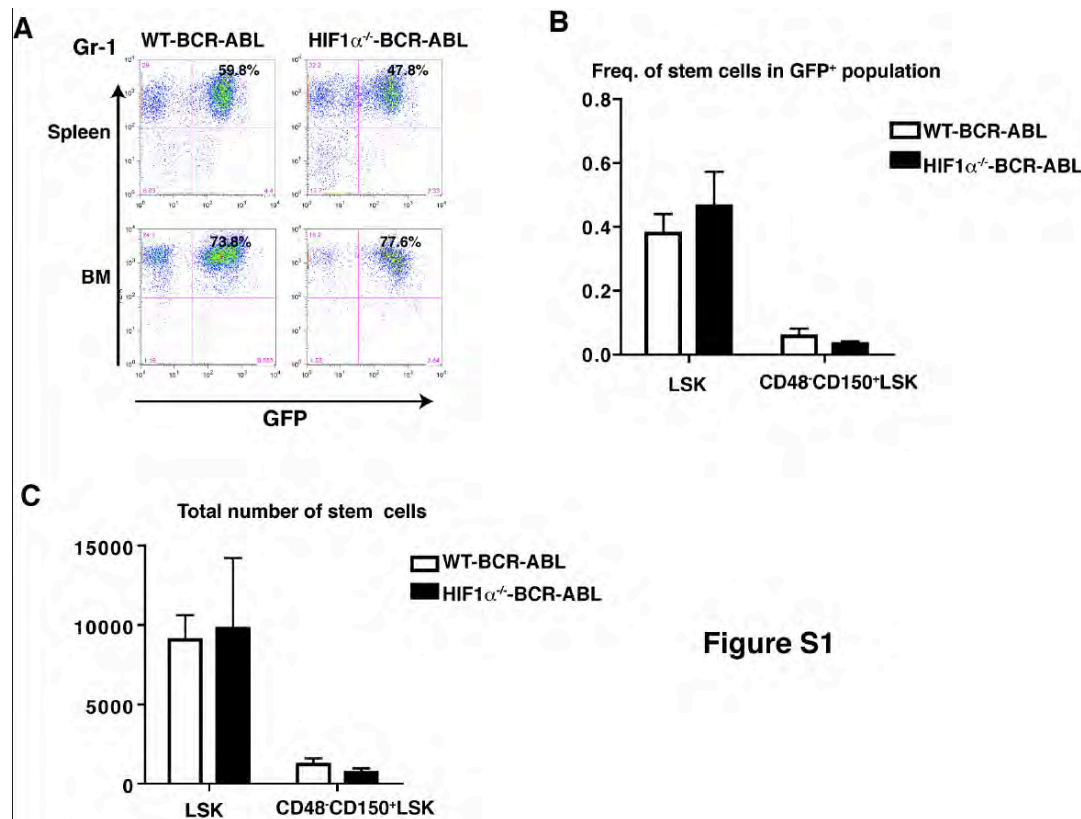


Figure S1

**Figure S1. The phenotype of primary WT and *HIF1* $\alpha^{-/-}$  CML mice.** (A) FACS analysis showed the percentages of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in spleen and BM from primary recipients of BCR-ABL transduced WT and *HIF1* $\alpha^{-/-}$  bone marrow cells at day 14. (B, C) The percentages and total numbers of LSCs (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells and GFP<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells) in bone marrow of primary recipient mice receiving BCR-ABL-transduced WT or *HIF1* $\alpha^{-/-}$  bone marrow cells were analyzed by FACS (n=6). Total numbers of LSCs were calculated by the percentage of stem cells x total number of bone marrow cells. Mean values are shown.

### CHAPTER III

#### **Tumor suppressor function of the Blk pathway in cancer stem cells of chronic myeloid leukemia**

**The work described in this chapter is accepted by Nature Genetics.**

#### **Abstract**

A therapeutic strategy for treating cancer is to target and eradicate cancer stem cells (CSCs) without harming their normal stem cell counterparts. The success of this approach relies on identification of molecular pathways that selectively regulate CSC function. Using BCR-ABL-induced chronic myeloid leukemia (CML) as a disease model for CSCs, we show that BCR-ABL down-regulates the *B lymphoid kinase (Blk)* gene through c-Myc in leukemia stem cells (LSCs) in CML mice and that Blk functions as a tumor suppressor in LSCs but does not affect normal hematopoietic stem cells (HSCs) or hematopoiesis. Blk suppresses LSC function through a pathway involving an upstream regulator, Pax5, and a downstream effector, p27. Inhibition of this Blk pathway accelerates CML development, whereas increased activity of the Blk pathway delays CML development. Our results demonstrate the feasibility of selectively targeting LSCs, an approach that should be applicable to other cancers.

## Introduction

Cancer stem cells (CSCs) are a cell population required for cancer initiation in many types of hematologic malignancies and some solid tumors, and must be eradicated to cure these cancers<sup>65-68</sup>. A recent study identified specific inhibitors of epithelial CSCs by high-throughput screening<sup>131</sup>, implying the existence of molecular pathways specific to CSCs that could serve as effective and selective therapeutic targets. We are interested in identifying genes that have essential roles in CSCs but not their normal stem cell counterparts. For example, previously we identified the *Alox5* gene as an important regulatory gene in leukemia stem cells (LSCs) but not normal hematopoietic stem cells (HSCs)<sup>85</sup>, demonstrating the feasibility of this approach.

We have been using BCR-ABL-induced chronic myeloid leukemia (CML) as a disease model because CML is a stem cell disease and we have previously identified LSCs for CML in mice<sup>63</sup>. CML is a clonal hematopoietic stem cell disorder associated with a reciprocal translocation between chromosomes 9 and 22 (t(9;22); also known as the Philadelphia chromosome); as a result, a chimeric BCR-ABL protein is formed that functions as a constitutively activated tyrosine kinase<sup>1,2,6</sup>. Although BCR-ABL kinase inhibitors are highly effective in treating chronic phase CML patients<sup>40,48,201</sup>, they do not efficiently kill LSCs<sup>62,63</sup>. Thus, new therapeutic strategies need to be developed. Because LSCs share many properties with normal HSCs, such as self-renewal, pluripotency and signaling<sup>65,89,90</sup>, it is important to develop therapies that only disturb the functions of LSCs but not normal HSCs.

In this study, we identify Blk, a Src family kinase, as a key regulator in CML LSCs. We show that Blk functions as a tumor suppressor in LSCs without affecting normal HSCs and mediates its inhibitory effect through a pathway involving an upstream regulator, Pax5, and a downstream effector, p27.

## Results

### **Blk has a tumor suppressor function in the induction of CML by BCR-ABL**

LSCs in CML are insensitive to BCR-ABL inhibitors<sup>62-64</sup>, suggesting that although some genes are activated or inactivated by BCR-ABL in LSCs, their expression is not affected by these inhibitors, i.e. their expression is BCR-ABL dependent but kinase independent. To identify genes regulated by BCR-ABL in LSCs, we compared gene expression profiles between normal LSK cells (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) and LSCs (BCR-ABL-expressing LSK) by DNA microarray as described previously<sup>85</sup>. We found that the *Blk* gene was down-regulated in LSCs, and, unexpectedly, this down-regulation was not significantly reversed by imatinib treatment (**Fig. 1A**). Real-time RT-PCR confirmed the down-regulation of *Blk* by BCR-ABL and the inability of imatinib to restore *Blk* expression in LSCs (**Fig. 1B**). Knockdown of BCR-ABL expression by an shRNA restored Blk expression in leukemia cells (**Fig. S1A, B**), suggesting that BCR-ABL down-regulates Blk in a kinase activity-independent manner.

The expression results raised the possibility that Blk suppresses CML development. To test this idea, we first studied the role of Blk in CML development using *Blk* homozygous knockout (*Blk*<sup>-/-</sup>) mice, which as expected did not express Blk

(**Fig. S2A**). If Blk suppresses CML development, we expect to observe accelerated disease development in the absence of Blk. **Fig. 1C** shows that recipients of BCR-ABL-transduced bone marrow cells ( $5 \times 10^5$  cells per recipient mouse) from 5-FU-treated *Blk*<sup>-/-</sup> donor mice developed CML and died significantly faster than did recipients of BCR-ABL-transduced WT bone marrow cells. Acceleration of CML due to Blk deficiency was more evident when a lower number of cells ( $1 \times 10^5$  cells) were transplanted. The accelerated disease phenotype correlated with a higher percentage and number of Gr-1<sup>+</sup> myeloid leukemia cells in peripheral blood (**Fig. 1D, E**), and with more severe infiltration of leukemic cells in the spleen (**Fig. 1F**). The lack of Blk did not affect BCR-ABL retroviral transduction efficiency (**Fig. S2B**) or homing of normal (**Fig. S2C**) and BCR-ABL-transduced (**Fig. S2D**) cells to bone marrow after transplantation.

To further demonstrate the tumor suppressor activity of Blk in CML development, we overexpressed Blk in donor bone marrow cells by transducing the cells with retrovirus expressing both BCR-ABL and Blk (**Fig. S3**). We found that overexpression of Blk significantly increased survival of CML mice (**Fig. 1G**), which correlated with a lower percentage of Gr-1<sup>+</sup> myeloid cells in peripheral blood (**Fig. 1H**) and decreased infiltration of leukemic cells in the spleen and lung (**Fig. 1I, J**).

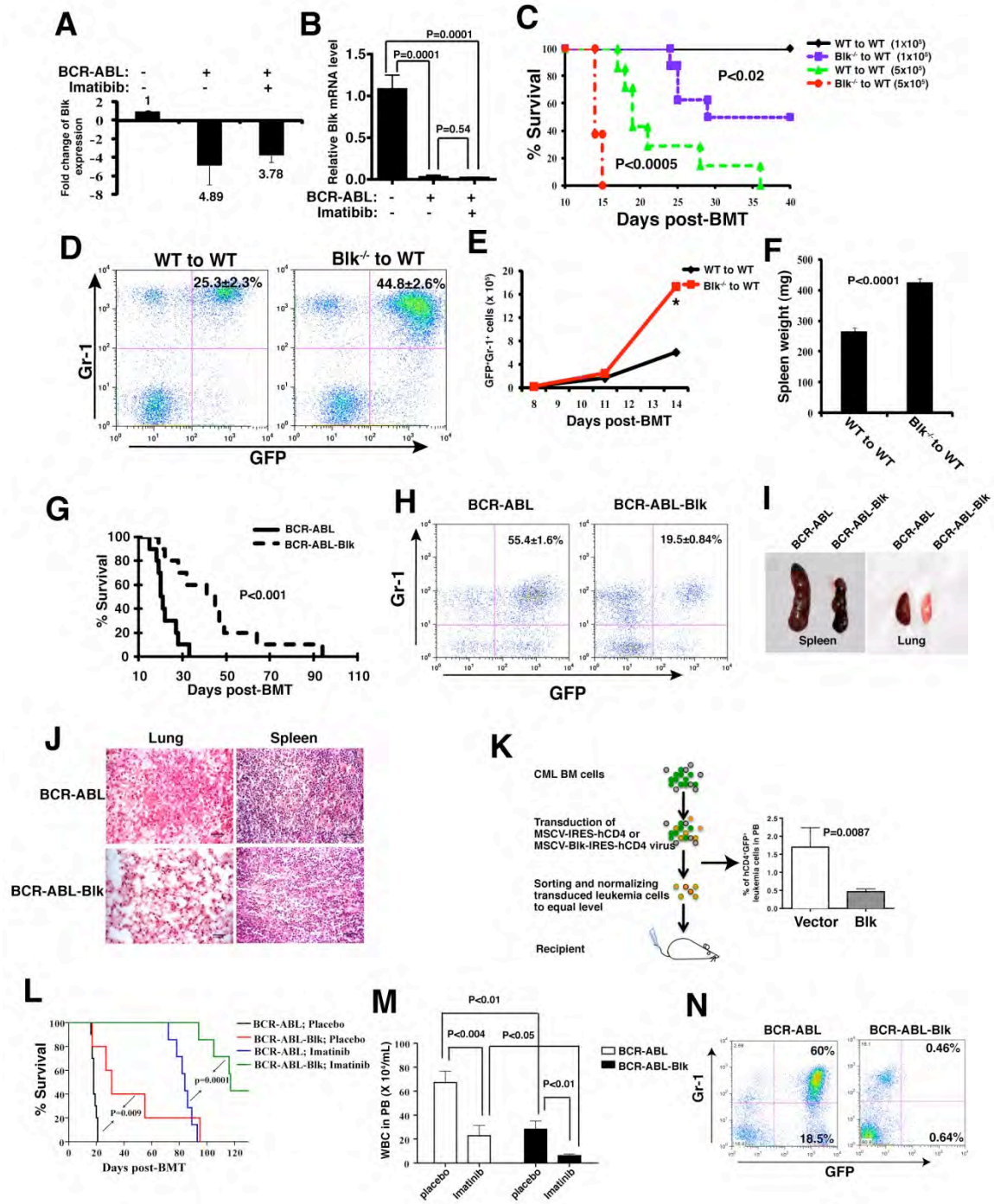
To determine whether Blk inhibits the progression of CML disease, we first induced CML and then transduced bone marrow cells, which contain established leukemic cells, with empty vector (MSCV-IRES-hCD4) or *Blk* (MSCV-Blk-IRES-hCD4). After sorting hCD4<sup>+</sup> cells by magnetic-activated cell sorting (MACS), we normalized and transplanted an equal number of GFP<sup>+</sup>hCD4<sup>+</sup> cells into recipient mice



(**Fig. 1K**), and monitored leukemia cell proliferation. We observed that the percentages of GFP<sup>+</sup>hCD4<sup>+</sup> leukemia cells in the two groups were initially similar, but Blk-expressing leukemia cells gradually decreased with time (**Fig. 1K**), indicating that Blk also inhibits progression of CML. Collectively, these results demonstrate that Blk strongly suppresses BCR-ABL-induced CML.

Suppression of LSCs in CML mice by Blk raised the possibility that restoration of Blk expression could synergize with a BCR-ABL kinase inhibitor in the treatment of CML. To test this idea, we induced CML in mice with BCR-ABL or BCR-ABL-Blk, and treated these mice with a placebo or imatinib. Either overexpression of Blk or imatinib treatment prolonged survival, as expected, but overexpression of Blk in combination of imatinib was much more effective, with about 40% of CML mice surviving longer than 130 days (**Fig. 1L**). This therapeutic effect correlated with lower white blood cell counts (**Fig. 1M**) and with the disappearance of leukemic cells (**Fig. 1N**).

**Figure 1. Blk suppresses the induction of CML by BCR-ABL.** (A) Microarray analysis showing expression of the *Blk* gene in LSCs of CML mice and upon imatinib treatment. Results are given as mean  $\pm$  SEM. (B) Real-time RT-PCR analysis of *Blk* expression in BCR-ABL-transduced LSCs from CML mice compared to GFP vector-transduced normal stem cells. Results are given as mean  $\pm$  SEM. (C) Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced bone marrow cells from WT (n=7) or *Blk*<sup>-/-</sup> (n=8) donor mice. (D) The percentage of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients of BCR-ABL-transduced bone marrow cells from WT or *Blk*<sup>-/-</sup> donor mice at day 11 after BMT. (P<0.002) (E) The total number of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients of *BCR-ABL*-transduced bone marrow cells from WT or *Blk*<sup>-/-</sup> donor mice at days 8, 11, and 14 after BMT. \*P<0.05 (F) Spleen weight in recipients of BCR-ABL-transduced *Blk*<sup>-/-</sup> and WT bone marrow cells at day 11 after BMT. Results are given as mean  $\pm$  SEM. (G) Kaplan-Meier survival curves for recipients of BCR-ABL (n=10) or BCR-ABL-Blk (n=10) transduced bone marrow cells. (H) The percentage of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients of BCR-ABL- or BCR-ABL-Blk-transduced donor bone marrow cells at day 15 after BMT. (P<0.001) (I) Gross appearance of the lungs and spleens of BCR-ABL- or BCR-ABL-Blk-transduced donor bone marrow cells at day 15 after BMT. (J) Photomicrographs of haematoxylin and eosin-stained lung and spleen sections from recipients of BCR-ABL- or BCR-ABL-Blk-transduced bone marrow cells (Scale bar = 100 $\mu$ m). (K) Leukemia cell growth in recipient mice transplanted with equal numbers of GFP<sup>+</sup>hCD4<sup>+</sup> cells. Results are given as mean  $\pm$  SEM. (L) Kaplan-Meier survival curves for recipients of BCR-ABL (n=7) or BCR-ABL-Blk (n=7) transduced bone marrow cells treated with a placebo or imatinib (P=0.0001). (M) Total number of white blood cells in peripheral blood of recipients of BCR-ABL- or BCR-ABL-Blk-transduced bone marrow cells at 1 week after the treatment with a placebo or imatinib. Results are given as mean  $\pm$  SEM. (N) FACS analysis showing the percentage of GFP<sup>+</sup> leukemia cells in recipients of BCR-ABL-Blk-transduced bone marrow cells at 8 weeks after imatinib treatment.



**Figure 1. Btk suppresses the induction of CML by BCR-ABL.**

### Blk suppresses LSCs

The down-regulation of *Blk* by BCR-ABL in LSCs and the ability of Blk to suppress CML development prompted us to test whether Blk suppresses proliferation of LSCs. **Fig. 2A** shows that the percentages of total LSCs and long-term (CD34<sup>-</sup>) or short-term (CD34<sup>+</sup>) LSCs (LT-LSCs or ST-LSCs, respectively) in bone marrow of recipients of BCR-ABL-transduced *Blk*<sup>-/-</sup> donor bone marrow cells were significantly higher than those in bone marrow of recipients of BCR-ABL-transduced WT donor bone marrow cells, indicating that Blk suppresses proliferation of LSCs. By contrast, Blk deficiency did not significantly alter the percentages of the myeloid progenitors CMP (common myeloid progenitor, Lin<sup>-</sup>Sca-1<sup>-</sup>Kit<sup>+</sup>CD34<sup>+</sup>FcyR2/3<sup>lo</sup>), GMP (granulocyte-macrophage progenitor, Lin<sup>-</sup>Sca-1<sup>-</sup>Kit<sup>+</sup>CD34<sup>+</sup>FcyR2/3<sup>hi</sup>), and MEP (megakaryocyte-erythroid progenitor, Lin<sup>-</sup>Sca-1<sup>-</sup>Kit<sup>+</sup>CD34<sup>-</sup>FcyR2/3<sup>lo</sup>) in bone marrow of CML mice (**Fig. 2A**).

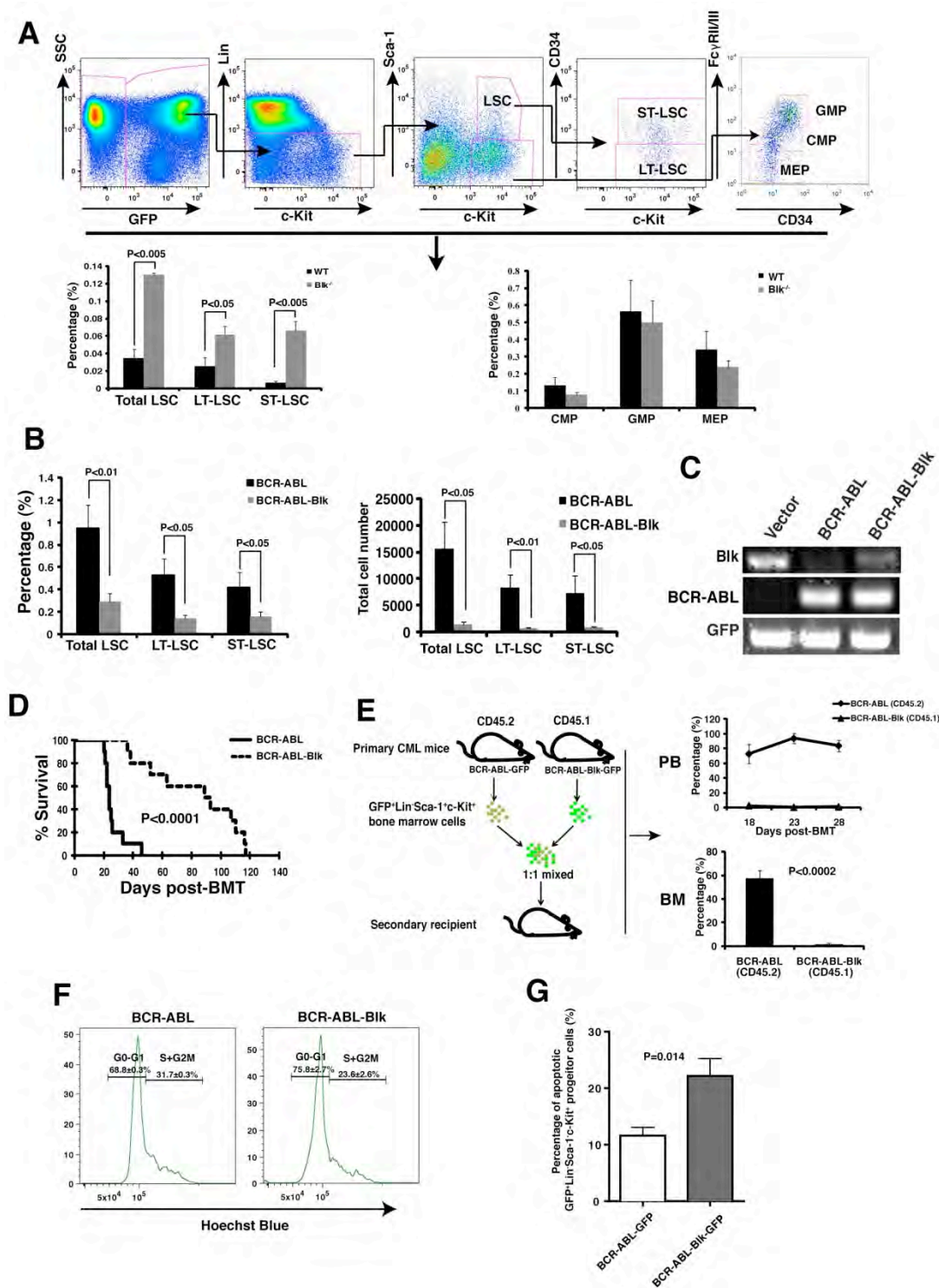
To confirm the inhibitory effect of Blk on LSCs, we tested whether Blk overexpression caused a reduction of LSCs in CML mice. We transduced bone marrow cells with BCR-ABL or BCR-ABL-Blk to induce CML, followed by transplantation into recipient mice. We observed that the percentages and numbers of total LSCs, LT-LSCs and ST-LSCs were significantly lower in recipients of BCR-ABL-Blk-transduced bone marrow cells than in recipients of BCR-ABL-transduced bone marrow cells (**Fig. 2B**), indicating that Blk suppressed proliferation of LSCs. To verify the ectopic expression of Blk, we sorted LSCs by FACS, isolated total RNA for RT-PCR analysis, and found that *Blk* expression was increased from an undetectable level in LSCs to a level close to that of endogenous *Blk* in normal HSCs (**Fig. 2C**). To further demonstrate the inhibitory

effect of Blk on LSCs, we tested their ability to transfer disease to secondary recipient mice. Bone marrow cells were transduced with BCR-ABL or BCR-ABL-Blk to induce primary CML, and then bone marrow cells were isolated from these CML mice and transferred into secondary recipient mice. **Fig. 2D** shows that Blk overexpression caused a significant delay of CML development in the secondary recipients (**Fig. 2D**), providing further evidence that Blk suppresses proliferation of LSCs. Consistently, *in vitro* colony-forming assay showed that Blk<sup>-/-</sup> LSCs generated much more colonies than WT LSCs; however, Blk-expressing LSCs gave rise to less colonies than WT LSCs (**Fig. S4**).

To more rigorously evaluate the inhibitory effect of Blk on LSC function, we examined whether Blk reduces the ability of LSCs to repopulate. LSCs were sorted by FACS from bone marrow of mice with primary CML induced by transplantation with BCR-ABL-transduced CD45.2 or BCR-ABL-Blk-transduced CD45.1 donor bone marrow cells. The sorted CD45.2 and CD45.1 LSCs were mixed in a 1:1 ratio, and transplanted into lethally irradiated recipient mice to induce secondary CML. At days 14, 23 and 28 after transplantation, fewer than 5% of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of the mice were CD45.1 leukemia cells that overexpressed Blk, whereas greater than 75%-80% of GFP<sup>+</sup>Gr-1<sup>+</sup> cells were CD45.2 leukemia cells that did not overexpress Blk (**Fig. 2E**). Consistent with these results, at day 28 after transplantation, the percentage of GFP<sup>+</sup>Gr-1<sup>+</sup> CD45.1 leukemia cells that overexpressed Blk was very low (**Fig. 2E**). The suppression of LSCs by Blk is explained, at least in part, by inhibition of cell cycle progression, as there were significantly fewer LSCs that overexpressed Blk in the S+G2M phase of the cell cycle compared to LSCs that did not overexpress Blk (**Fig. 2F**).

In addition, we observed increased apoptosis in LSCs from recipients of BCR-ABL-Blk-transduced bone marrow cells (**Fig. 2G**), suggesting that Blk also inhibits proliferation and induces apoptosis of LSCs.

**Figure 2. Blk suppresses LSCs.** (A) The percentages of total LSCs (GFP<sup>+</sup>LS<sup>+</sup>K<sup>+</sup>), long-term (LT)-LSCs (GFP<sup>+</sup>LS<sup>+</sup>K<sup>+</sup>CD34<sup>-</sup>), short-term (ST)-LSCs (GFP<sup>+</sup>LS<sup>+</sup>K<sup>+</sup>CD34<sup>+</sup>), CMP (GFP<sup>+</sup>LS<sup>-</sup>K<sup>+</sup>CD34<sup>+</sup>FcyR2/3<sup>lo</sup>), GMP (GFP<sup>+</sup>LS<sup>-</sup>K<sup>+</sup>CD34<sup>+</sup>FcyR2/3<sup>hi</sup>), and MEP (GFP<sup>+</sup>LS<sup>-</sup>K<sup>+</sup>CD34<sup>+</sup>FcyR2/3<sup>lo</sup>) in bone marrow of recipients of BCR-ABL-transduced *Blk*<sup>-/-</sup> donor bone marrow cells (n=9) were compared with those in bone marrow of recipients of BCR-ABL-transduced WT donor bone marrow cells (n=5) at day 11 after BMT. Mean values ( $\pm$  SEM) are shown. (B) The percentages and numbers of total LSCs, LT-LSCs and ST-LSCs in bone marrow of recipients of BCR-ABL- or BCR-ABL-*Blk*-transduced bone marrow cells were analyzed at day 15 after BMT. Mean values ( $\pm$  SEM) are shown (n=5). (C) RT-PCR analysis of expression of *Blk*, *BCR-ABL*, and *GFP* in LSCs. Bone marrow cells transduced with retrovirus expressing *GFP* alone (vector), *BCR-ABL/GFP*, or *BCR-ABL/Blk/GFP* were transplanted into recipient mice to induce CML. 2 weeks after BMT, LSCs were sorted by FACS from bone marrow of the mice for isolation of total RNA for RT-PCR analysis. (D) Kaplan-Meier survival curves for secondary CML mice. Bone marrow cells from primary CML mice induced by *BCR-ABL* or *BCR-ABL-Blk* were collected at day 15 after BMT, and equal number of the cells from each group were transplanted into lethally irradiated secondary recipient mice (n=10 for each group). (E) Competitive repopulation assay. 10<sup>3</sup> sorted-LSCs from bone marrow of primary CML mice induced by transplanting BCR-ABL-transduced CD45.2 or BCR-ABL-*Blk*-transduced CD45.1 donor bone marrow cells were mixed at 1:1 ratio, followed by transplantation into lethally irradiated recipients mice. The percentages of *BCR-ABL-Blk* expressing (CD45.1) cells in peripheral blood and bone marrow were compared with those of *BCR-ABL*-expressing (CD45.2) cells at 2, 3, and 4 weeks after BMT (n=3 for each time point). (F) The cell cycle analysis of LSCs from bone marrow of CML mice induced by BCR-ABL or BCR-ABL-*Blk* (n=5 for each group). Bone marrow cells were stained with Hoechst Blue, and DNA contents in the S+G2M phase of the cell cycle in LSCs were examined by FACS (P<0.05). (G) Percentage of apoptotic GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells in bone marrow from CML mice at day 14 after BMT. Mean values ( $\pm$  SEM) are shown.



**Figure 2. Btk suppresses LSCs.**



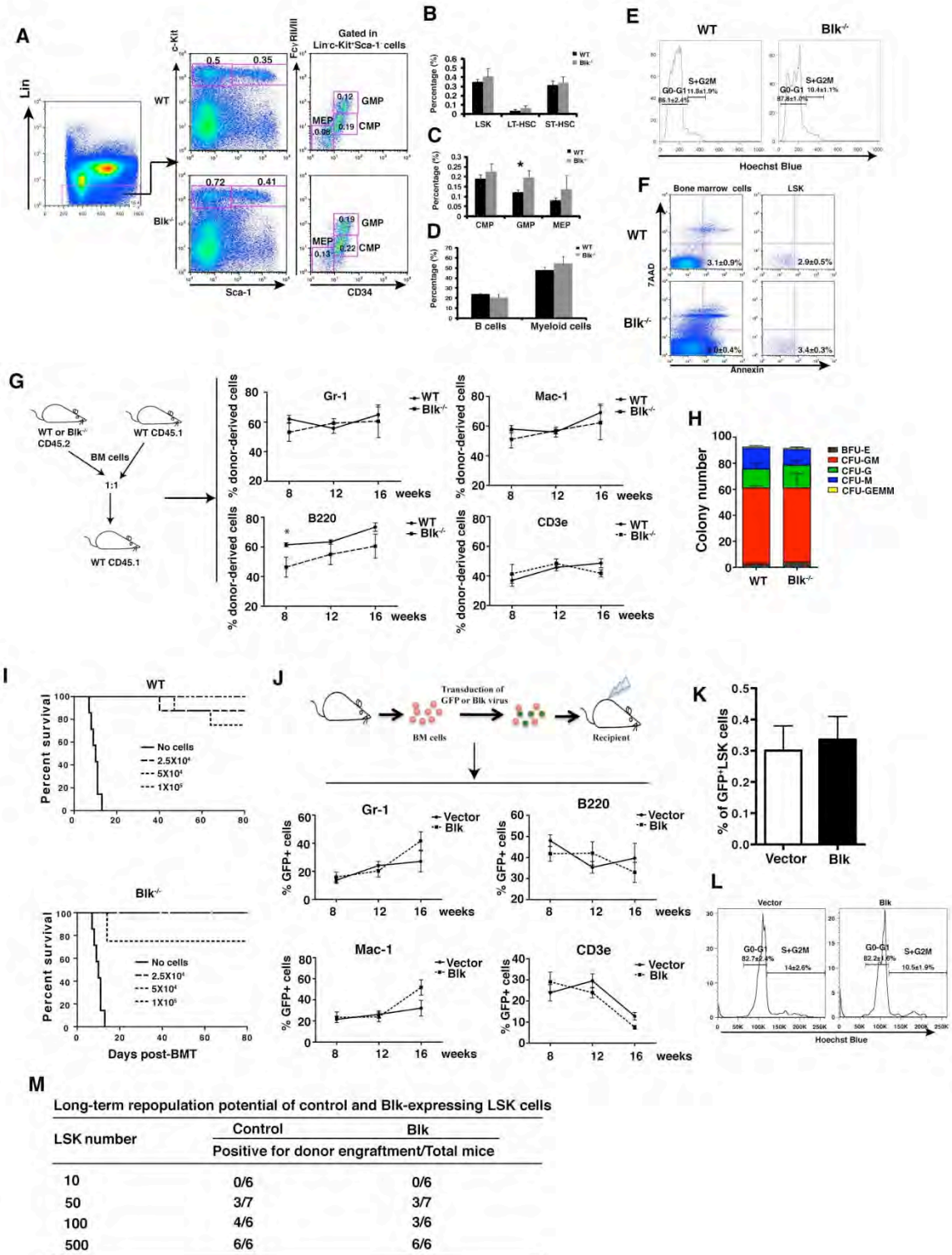
### **Blk does not suppress normal hematopoietic stem cells**

The finding that Blk suppresses proliferation and induces apoptosis of LSCs prompted us to ask whether Blk has a similar effect on normal HSCs. We examined the effect of Blk on normal hematopoiesis and HSCs. **Fig. 3A-C** show that the percentages of total LSK, LT-HSCs, ST-HSCs, CMP and MEP in bone marrow of *Blk*<sup>-/-</sup> and WT mice were similar, although the percentage of GMP was higher in *Blk*<sup>-/-</sup> (0.19%) than in WT (0.12%) mice. Notably, however, there was no significant difference in more mature myeloid cells (Gr-1<sup>+</sup>Mac-1<sup>+</sup>) in bone marrow of *Blk*<sup>-/-</sup> and WT mice (**Fig. 3D**). We also found that Blk deficiency did not affect cell cycle progression (**Fig. 3E**) or apoptosis (**Fig. 3F**) of LSK cells. These results indicate that Blk does not suppress normal HSCs or hematopoiesis. To examine whether Blk deficiency affects the function of normal HSCs, we performed a competitive repopulation assay. 2x10<sup>5</sup> bone marrow cells from WT or *Blk*<sup>-/-</sup> mice (CD45.2) were transplanted into each lethally irradiated WT recipient (CD45.1) along with an equal number of WT competitor cells (CD45.1). The lineage contribution of WT or *Blk*<sup>-/-</sup> cells in recipient mice was evaluated at 8, 12 and 16 weeks after transplantation. We observed similar percentages of donor-derived myeloid (Gr-1<sup>+</sup> and Mac-1<sup>+</sup>) and T lymphoid cells (CD4<sup>+</sup> and CD8<sup>+</sup>) (**Fig. 3G**), indicating that Blk did not affect the function of normal HSCs. Although *Blk* deficiency affected the levels of B cells (B220<sup>+</sup>) (**Fig. 3G**), this effect is likely due to the known role of *Blk* in B cell development<sup>154</sup>. We also performed colony-forming assay to examine the effect of *Blk* on progenitor cell function in vitro using sorted LSK cells from *Blk*<sup>-/-</sup> and WT mice bone marrow. Similar numbers and types of colonies were formed in the presence and absence

of Blk (**Fig. 3H**). Further, we analyzed the abilities of WT and *Blk*<sup>-/-</sup> bone marrow cells to rescue recipient mice from lethal irradiation. **Fig. 3I** shows that there was no significant difference in the ability of WT and *Blk*<sup>-/-</sup> bone marrow cells to rescue lethally irradiated mice.

To provide additional evidence for the role of Blk in regulation of the function of HSCs, we tested whether overexpression of Blk suppresses proliferation of HSCs. We transduced bone marrow cells from WT mice with Blk-GFP or GFP retrovirus, followed by transplantation into recipient mice (**Fig. 3J**). The lineage contribution of GFP or Blk-GFP cells in recipient mice was evaluated at 8, 12 and 16 weeks after transplantation. The percentages of mature myeloid cells (GFP<sup>+</sup>Gr-1<sup>+</sup>/Mac-1<sup>+</sup>), B-lymphoid cells (GFP<sup>+</sup>B220<sup>+</sup>), and T cells (GFP<sup>+</sup>CD3e<sup>+</sup>) in peripheral blood of recipients of Blk-GFP- or GFP-transduced marrow cells were similar (**Fig. 3J**), and the percentages of GFP<sup>+</sup>LSK cells in bone marrow of recipients of Blk-GFP- or GFP-transduced marrow cells at 16 weeks after transplantation were also similar (**Fig. 3K**). In addition, there was no significant difference in cell cycle progression between Blk-GFP- and GFP-transduced bone marrow cells (**Fig. 3L**). Next, we conducted an in vivo limiting dilution analysis. 16 weeks after transplantation, GFP<sup>+</sup>LSK cells were sorted from recipients of Blk-GFP or GFP transduced marrow cells, and were injected into secondary recipients at different doses (10, 50, 100 or 500 cells). After 12 weeks, we analyzed the GFP<sup>+</sup> cells. Poisson statistics showed no significant difference in the frequency of long-term repopulation ability among control and Blk-transduced cells (**Fig. 3M**). These results indicate that Blk overexpression does not affect the long-term repopulation activity of HSCs.

**Figure 3 *Blk* does not inhibit hematopoiesis or normal HSCs.** (A-C) FACS analysis of HSCs, CMP, GMP and MEP cell populations in bone marrow of WT (n=3) or *Blk*<sup>-/-</sup> (n=4) mice. (B) The percentages of LSK, LT-HSCs, and ST-HSCs in bone marrow of WT or *Blk*<sup>-/-</sup> mice. (C) The percentages of CMP, GMP, and MEP cells in bone marrow of WT (n=3) or *Blk*<sup>-/-</sup> (n=4) mice. (D) The percentages of myeloid (Gr-1<sup>+</sup>Mac-1<sup>+</sup>) and lymphoid (B220<sup>+</sup>IgM<sup>+</sup>) cells in bone marrow of WT or *Blk*<sup>-/-</sup> mice. (E) Cell cycle analysis of LSK cells in bone marrow of WT or *Blk*<sup>-/-</sup> mice. (F) Apoptosis analysis of bone marrow cells and LSK cells from WT (n=3) or *Blk*<sup>-/-</sup> (n=4) through staining with Annexin V and 7AAD. (G) Competitive repopulation assay. FACS analysis showed donor contribution of different cell lineages in recipient mice at 8, 12 and 16 weeks after transplantation. \* P<0.05 (H) Colony forming assay showed equivalent colony numbers and types between WT and *Blk*<sup>-/-</sup> group. (I) Irradiation rescue assay. Three doses (1x10<sup>5</sup>, 5x10<sup>5</sup>, 2.5x10<sup>4</sup>) of WT or *Blk*<sup>-/-</sup> BM cells were injected into lethally irradiated recipients, and survival curves for these three groups were compared. (J) In vivo long-term reconstitution analysis. FACS analysis showed the lineage contribution in peripheral blood from recipients of *Blk* and vector transduced bone marrow cells at 8, 12, 16 weeks after transplantation. (K) The percentages of LSK cells in the GFP<sup>+</sup> population of bone marrow from recipients of vector and *Blk* transduced BM cells at 16 weeks after transplantation. (L) Cell cycle analysis of LSK cells from bone marrow of recipients transplanted with *GFP* or *Blk/GFP* transduced bone marrow cells. (P=0.86 for G0-G1; P=0.2 for S+G2M). (M) In vivo limiting dilution analysis. 16 weeks after BMT, GFP+LSK cells were sorted from recipients of *Blk-GFP* or *GFP* transduced marrow cells, and were injected into secondary recipients in different doses (10, 50, 100 or 500 cells per mouse). After 12 weeks, the GFP<sup>+</sup> cells were examined by FACS. Poisson statistics showed no significant difference in the frequency of long-term repopulation among control and *Blk*-transduced cells.



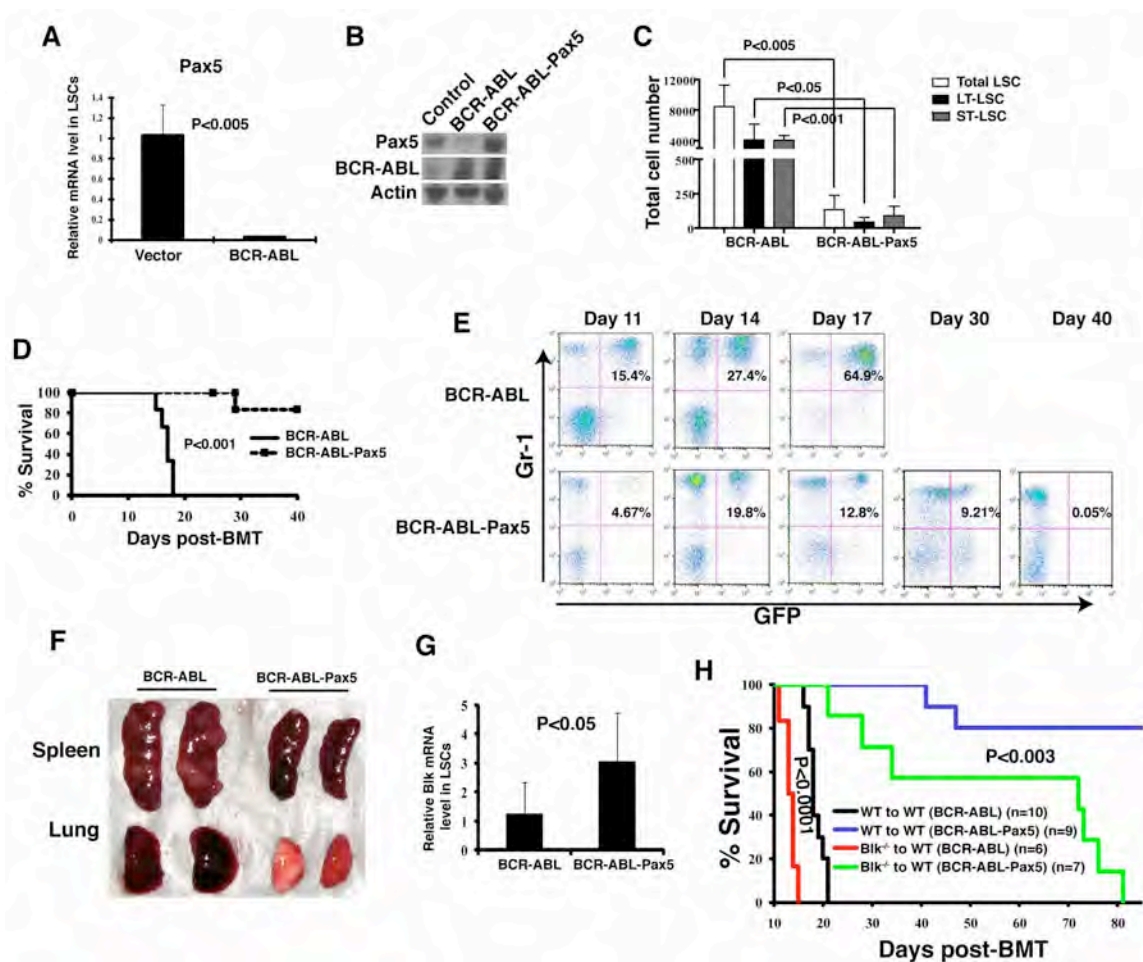
**Figure 3. Blk does not inhibit hematopoiesis or normal HSCs.**

### **Pax5 is an upstream regulator of *Blk* in LSCs**

We next studied the underlying mechanisms by which *Blk* has an inhibitory effect on LSCs but not normal HSCs. Pax5 is known to bind to the *Blk* promoter and stimulate *Blk* expression<sup>202</sup>. We therefore considered the possibility that the down-regulation of *Blk* expression by BCR-ABL in LSCs is mediated through Pax5. In support of this idea, we found that BCR-ABL markedly down-regulated *Pax5* expression in LSCs (**Fig. 4A**). To test whether Pax5 suppresses proliferation of LSCs and CML development, we generated a retroviral construct that co-expressed BCR-ABL and Pax5 (**Fig. 4B**). We transduced bone marrow cells with BCR-ABL or BCR-ABL-Pax5, followed by transplantation into recipient mice. Fourteen days later, bone marrow cells from CML mice were analyzed for the percentages and numbers of LSCs. The results of **Fig. 4C** show that Pax5 overexpression caused a marked decrease in total, LT- and ST-LSCs. We next compared survival between the two transplantation groups. All recipients of BCR-ABL-transduced bone marrow cells died of CML within 3 weeks, whereas fewer than 20% of the recipients of BCR-ABL-Pax5-transduced bone marrow cells developed CML and died (**Fig. 4D**), which correlated with lower, gradually decreased percentages of myeloid leukemia cells in peripheral blood during the course of the disease (**Fig. 4E**), and with less severe splenomegaly and leukemic cell infiltration in the spleen and lung (**Fig. 4F**). We also found that ectopically expressed Pax5 caused an increase of *Blk* expression in LSCs (**Fig. 4G**), supporting the idea that Pax5 functions upstream of *Blk* to mediate the down-regulation of *Blk* by BCR-ABL. To further test this idea, we transduced bone marrow cells from *Blk*<sup>-/-</sup> or WT mice with BCR-ABL-Pax5 or BCR-ABL alone, followed

by transplantation into recipient mice. If Pax5 is upstream of *Blk*, the delay of CML development by Pax5 should be prevented in the absence of *Blk*. We found that compared to the accelerated CML development in recipients of *Blk*<sup>-/-</sup> bone marrow cells transduced by BCR-ABL alone (**Fig. 4H**), recipients of *Blk*<sup>-/-</sup> bone marrow cells transduced with BCR-ABL-Pax5 died of CML much more slowly, although these mice developed CML significantly faster than recipients of BCR-ABL-Pax5-transduced WT bone marrow cells. These results suggest that *Blk* is one but not only the downstream functional target gene of Pax5 in LSCs.

**Figure 4 *Pax5* is an upstream partner of *Blk* in LSCs.** (A) Real time RT-PCR analysis showing expression of *Pax5* in LSCs as compared to normal HSCs. Results are given as mean  $\pm$  s.e.m. (B) Western blot analysis showing expression of Pax5 and BCR-ABL in 293T cells transfected with BCR-ABL and BCR-ABL-Pax5. (C) FACS analysis of the numbers of total LSCs, LT-LSCs, and ST-LSCs from recipients of BCR-ABL- or BCR-ABL-Pax5-transduced BM cells. Results are given as mean  $\pm$  s.e.m. (D) Kaplan-Meier survival curves for recipients of BCR-ABL- (n=7) or BCR-ABL-Pax5- (n=6) transduced bone marrow cells. (E) FACS analysis showing the percentages of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients of BCR-ABL- or BCR-ABL-Pax5-transduced bone marrow cells at days 11, 14, 17, 30, and 40 after BMT, and gradual disappearance of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients of BCR-ABL-Pax5-transduced bone marrow cells but not in recipients of BCR-ABL-transduced bone marrow cells. (F) Gross appearance of the lungs and spleens of recipients of BCR-ABL- or BCR-ABL-Pax5-transduced donor bone marrow cells at day 14 after BMT. (G) Real time RT-PCR analysis monitoring *Blk* expression in LSCs from bone marrow of recipients of BCR-ABL- and BCR-ABL-Pax5-transduced bone marrow cells. Bone marrow cells from mice with CML induced by *BCR-ABL* or *BCR-ABL-Pax5* were cultured under stem cell conditions for 6 days, and LSCs were sorted by FACS for isolation of total RNA for real time PCR analysis. (H) Kaplan-Meier survival curves for recipients of BCR-ABL- or BCR-ABL-Pax5-transduced WT or *Blk*<sup>-/-</sup> bone marrow cells.



**Figure 4** *Pax5* is an upstream partner of *Blk* in LSCs.



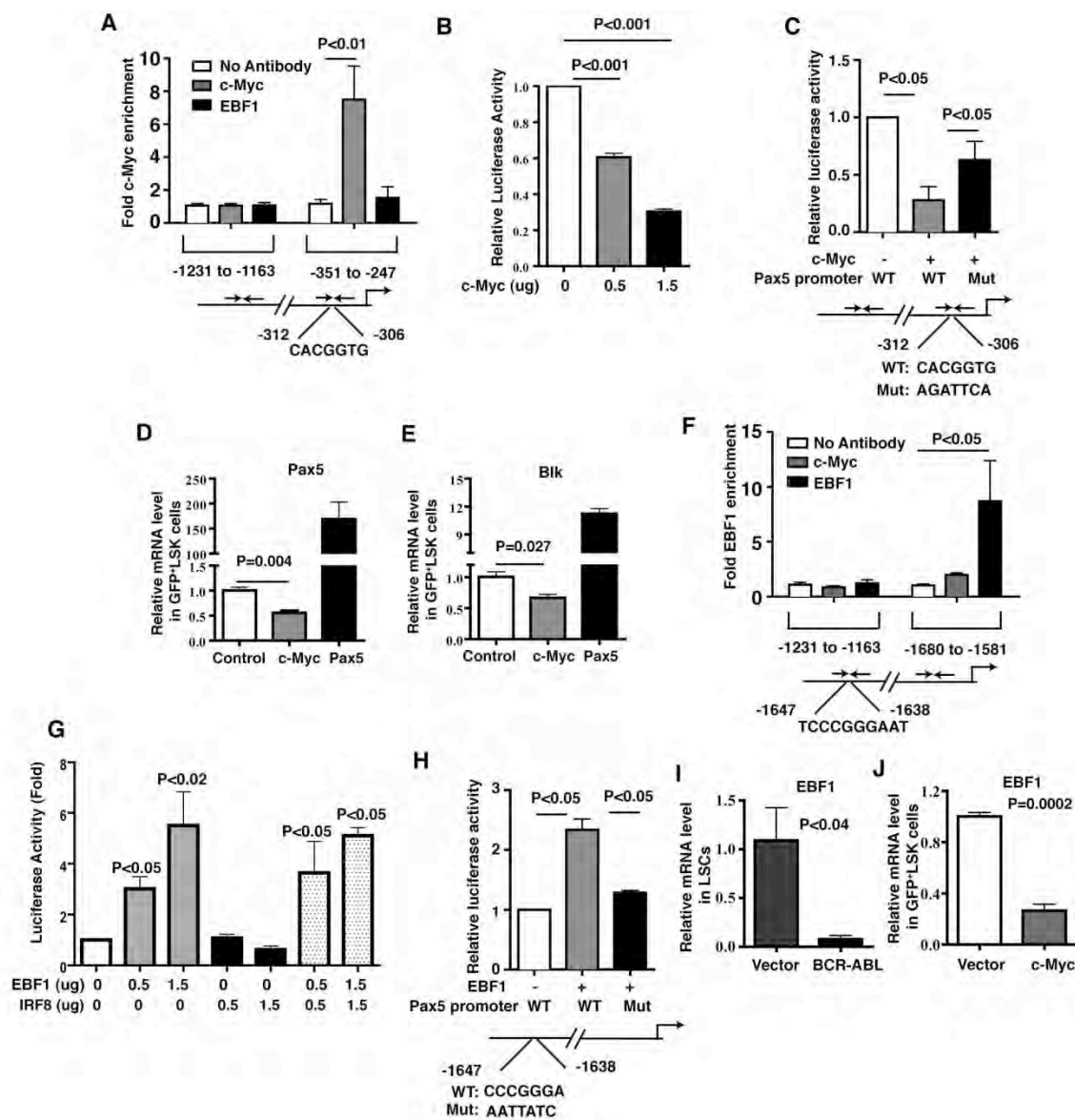
### **c-Myc and EBF1 mediate down-regulation of *Pax5* by BCR-ABL**

We next studied how BCR-ABL down-regulates *Pax5* expression. Previous studies have shown that BCR-ABL induces c-Myc expression<sup>203-205</sup>, and analysis of the *Pax5* promoter region revealed a consensus c-Myc binding motif at -312 basepairs upstream of the transcription start site (**Fig. S5A**). ChIP analysis demonstrated that c-Myc directly bound to this region, but not to a region further upstream (**Fig. 5A**). Therefore, we investigated whether BCR-ABL down-regulates *Pax5* through c-Myc using a luciferase assay for monitoring *Pax5* promoter activity. **Fig. 5B** shows that expression of c-Myc caused a reduction of *Pax5* promoter activity in a dose dependent manner in NIH3T3 cells, indicating that c-Myc suppresses *Pax5* expression at the transcriptional level. In addition, we mutated the c-Myc binding site in the *Pax5* promoter, and found that the suppression of luciferase activity by c-Myc was markedly rescued (**Fig. 5C**). These data indicate that c-Myc directly binds to and suppresses *Pax5* expression at the transcriptional level. To determine whether c-Myc down-regulates *Pax5* expression in HSCs, we transduced bone marrow cells with a retrovirus expressing c-Myc (**Fig. S5B**) or, as a control, Pax5. Real time RT-PCR analysis showed that c-Myc significantly inhibited expression of *Pax5* and *Blk* in LSK cells (**Fig. 5D** and **5E**), whereas *Pax5* dramatically enhanced *Blk* expression in these cells (**Fig. 5E**). These results indicate that the down-regulation of *Pax5* by BCR-ABL is mediated by c-Myc.

Previous studies have suggested that the transcription factor EBF1 binds to the *Pax5* promoter and stimulates *Pax5* expression<sup>206,207</sup>, and our microarray results indicated that *EBF1* was significantly downregulated in LSCs (**Fig. S6**). To investigate

the role of EBF1, we conducted a ChIP analysis and found that EBF1 directly bound to *Pax5* promoter within a region from -1638 to -1647 (**Fig. 5F**), consistent with previous results<sup>206,207</sup>. Using a *Pax5* promoter-luciferase reporter, we found that expression of EBF1 increased luciferase activity (**Fig. 5G**). Because interferon regulatory factor 8 (IRF8) is known to regulate expression of *EBF1* and *Pax5*<sup>208,209</sup>, we also tested whether IRF8 regulates *Pax5* expression by itself or synergistically with EBF1. However, IRF8 had no effect on *Pax5* promoter activity (**Fig. 5G**). We mutated the EBF1 binding site in the *Pax5* promoter, and found that the increased luciferase activity by EBF1 was markedly inhibited (**Fig. 5H**). It remained possible that c-Myc also down-regulates *EBF1* expression resulting in decreased *Pax5* expression. Therefore, we asked whether BCR-ABL and c-Myc down-regulate EBF1 expression in LSCs. Real time RT-PCR analysis showed that BCR-ABL down-regulated *EBF1* expression in LSCs (**Fig. 5I**), and c-Myc down-regulated *EBF1* expression in LSK cells (**Fig. 5J**). Thus, down-regulation of *Pax5* by BCR-ABL is mediated by c-Myc and EBF1.

**Figure 5 c-Myc and EBF1 regulate *Pax5* expression.** (A) ChIP assay showed c-Myc directly bound to the *Pax5* promoter. Results were shown as mean  $\pm$  s.e.m. (B) A *Pax5* promoter luciferase reporter construct was cotransfected with empty vector or c-Myc plasmid into NIH3T3 cells. Cell extracts were analyzed for luciferase activity. Results were shown as mean  $\pm$  s.e.m.. (C) Luciferase assay showed mutant c-Myc binding site in the *Pax5* promoter restored the luciferase activity. Results were shown as mean  $\pm$  s.e.m. (D and E) Real time RT-PCR analysis monitoring *Pax5* and *Blk* expression in c-Myc-expressing or *Pax5*-expressing LSK cells. Results were shown as mean  $\pm$  s.e.m. (F) ChIP assay showed EBF1 directly bind to the *Pax5* promoter. Results were shown as mean  $\pm$  s.e.m. (G) A *Pax5* promoter luciferase reporter construct was cotransfected with empty vector, *EBF1*, or *IRF8* plasmids into NIH3T3 cells. Cell extracts were analyzed for luciferase activity. Results were shown as mean  $\pm$  s.e.m. (H) Luciferase assay showed mutant EBF1 binding site in the *Pax5* promoter rescued the luciferase activity. Results were shown as mean  $\pm$  s.e.m. (I) Real time RT-PCR analysis monitoring *EBF1* expression by *BCR-ABL* in LSCs as compared to normal HSCs. Results are given as mean  $\pm$  s.e.m. (J) Real time RT-PCR analysis monitoring *EBF1* expression in c-Myc-expressing LSK cells. Results are given as mean  $\pm$  s.e.m.



**Figure 5** c-Myc and EBF1 regulate *Pax5* expression

### **p27 functions downstream of Blk to suppress proliferation of LSCs**

Next, we attempted to identify genes required for Blk to suppress LSC proliferation and CML development. The mammalian cyclin-dependent kinase inhibitor (CDKI) p27 is a negative cell cycle regulator that blocks the G1 to S phase transition<sup>210</sup>. BCR-ABL down-regulates p27 expression through multiple mechanisms<sup>211-214</sup>. Because Blk inhibits cell cycle progression of LSCs, we considered the possibility that Blk functions through p27. To test this idea, we first compared the levels of p27 in 293T cells transfected with BCR-ABL alone or with both BCR-ABL and Blk. We found that BCR-ABL down-regulated p27 and Blk restored the level of p27 expression (**Fig. 6A**). The inhibition of Skp2 by Blk was confirmed by real time RT-PCR (**Fig. S7A**). Notably, BCR-ABL and Blk did not alter the levels of other cell cycle regulators such as p21 and Cdk2 (**Fig. 6A**).

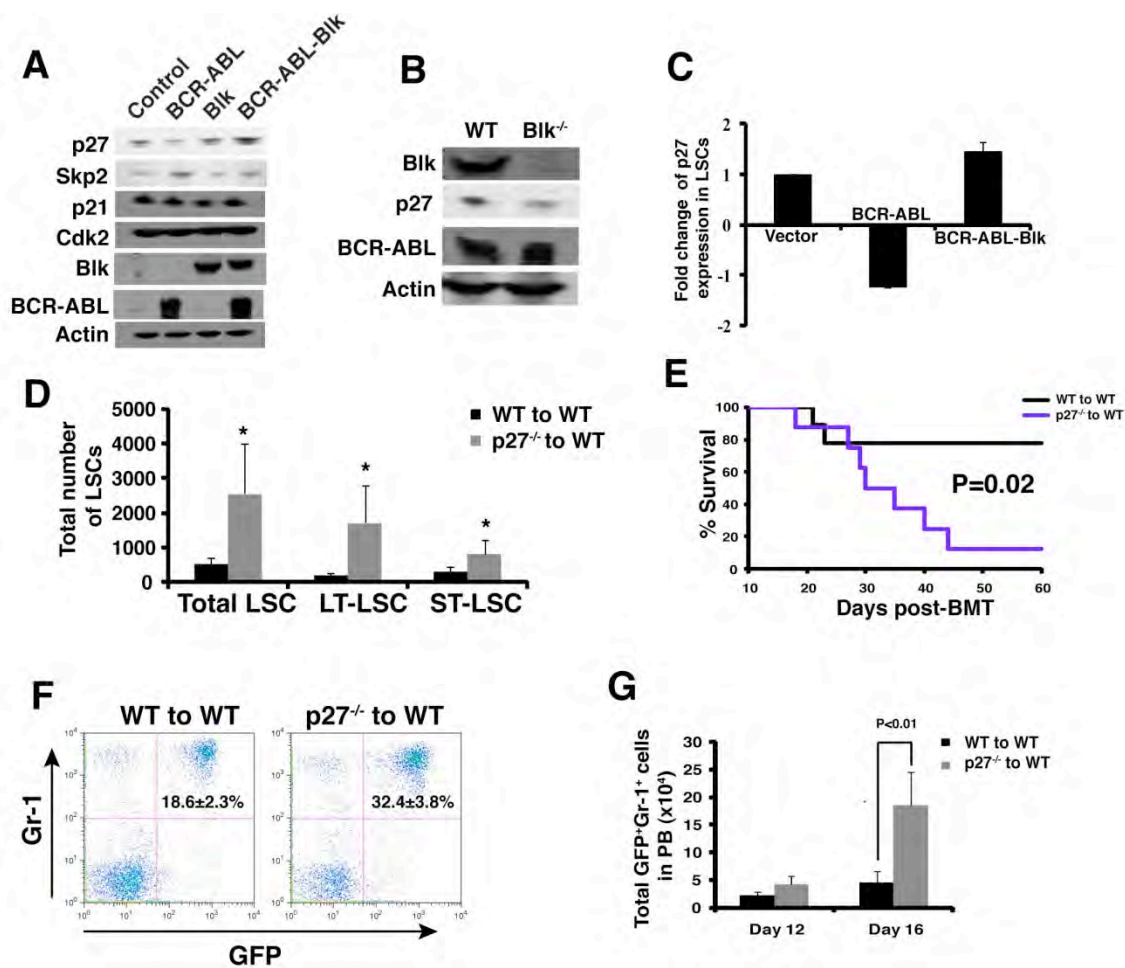
To confirm that BCR-ABL functions through Blk to reduce *p27* expression, we transduced WT or *Blk*<sup>-/-</sup> bone marrow cells with BCR-ABL. We found that p27 expression was significantly lower in the absence of Blk (**Fig. 6B** and **Fig. S7B**). Conversely, we overexpressed *Blk* in LSCs both to verify that Blk increases *p27* expression and to identify other Blk target genes. Toward this end, bone marrow cells were transduced with GFP, BCR-ABL-GFP or BCR-ABL-Blk-GFP, followed by transplantation into recipient mice. Fourteen days after transplantation, bone marrow cells were isolated and LSCs cells were sorted by FACS for isolation of total RNA for DNA microarray analysis. We found that BCR-ABL down-regulated *p27* expression, which

was reversed by *Blk* overexpression (**Fig. 6C**). We also identified many other genes that were significantly up- or down-regulated by *Blk* in LSCs (**Table S1 and S2**).

We next tested whether p27 could suppress LSC proliferation and CML development. We transduced WT or *p27*<sup>-/-</sup> bone marrow cells with BCR-ABL, followed by transplantation of a relatively small number of transduced cells (1x10<sup>5</sup> cells per recipient). After 14 days, bone marrow cells from CML mice were analyzed for the number of LSCs. **Fig. 6D** shows that p27 deficiency caused a marked increase in total, LT- and ST-LSCs. Significantly, only 20% of recipients of BCR-ABL-transduced WT bone marrow cells died by 60 days after transplantation, whereas 90% of recipients of BCR-ABL-transduced *p27*<sup>-/-</sup> bone marrow cells died by 45 days after the transplantation (**Fig. 6E**). This accelerated CML development in the absence of p27 correlated with a higher percentage and number of myeloid leukemia cells in peripheral blood (**Fig. 6F,G**).

The regulation of *p27* by *Blk* in cell cycle progression was further demonstrated in cultured bone marrow cells from CML mice. Consistent with the results in 293T cells, *Blk* overexpression increased expression of p27 but not CDK2 and Cyclin E (**Fig. S7C**). Collectively, these results indicate that p27 functions downstream of *Blk* to suppress LSC proliferation and CML development. By contrast, although an elevated *p27* expression was found in *Blk*-expressing HSCs (**Fig. S7D**), the percentage of HSCs were similar in bone marrow of mice receiving control and *Blk*-transduced marrow cells (**Fig. 3G**), indicating that p27 does not suppress proliferation of normal HSCs.

**Figure 6. p27 is a downstream partner of Blk in LSCs.** (A) Western blot analysis of the expression of p27, p21, Cdk2 and Skp2 in 293T cells after transfection with BCR-ABL, Blk or BCR-ABL-Blk. (B) Western blot analysis monitoring p27 expression. *BCR-ABL*-transduced bone marrow cells from WT or *Blk*<sup>-/-</sup> mice were grown in Whitlock-Witte culture for 7 days, and protein lysates were isolated for comparing p27 expression regulated by BCR-ABL in the presence and absence of BCR-ABL by Western blotting. (C) Microarray analysis showing *p27* expression in vector-, BCR-ABL- and BCR-ABL-Blk-transduced LSCs. Mean values ( $\pm$  SEM) are shown. (D) The total numbers of total LSCs, LT-LSCs, and ST-LSCs in bone marrow of recipients of BCR-ABL-transduced *p27*<sup>-/-</sup> (n=4) and BCR-ABL-transduced WT (n=3) donor bone marrow cells at day 14 after BMT. Mean values ( $\pm$  SEM) are shown. (\*P<0.05) (E) Kaplan-Meier survival curves for recipients of BCR-ABL-transduced bone marrow cells from WT or *p27*<sup>-/-</sup> donor mice (P=0.02; n=8 for each group). (F) FACS analysis showing the percentages of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients of *BCR-ABL*-transduced bone marrow cells from WT or *p27*<sup>-/-</sup> donor mice at day 12 after BMT. (P<0.02) (G) The total numbers of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients BCR-ABL-transduced bone marrow cells from WT or *p27*<sup>-/-</sup> donor mice at days 12 and 16 after BMT. Mean values ( $\pm$  SEM) are shown.



**Figure 6. p27 is a downstream partner of Blk in LSCs.**



### Suppression of CML does not require Blk kinase activity

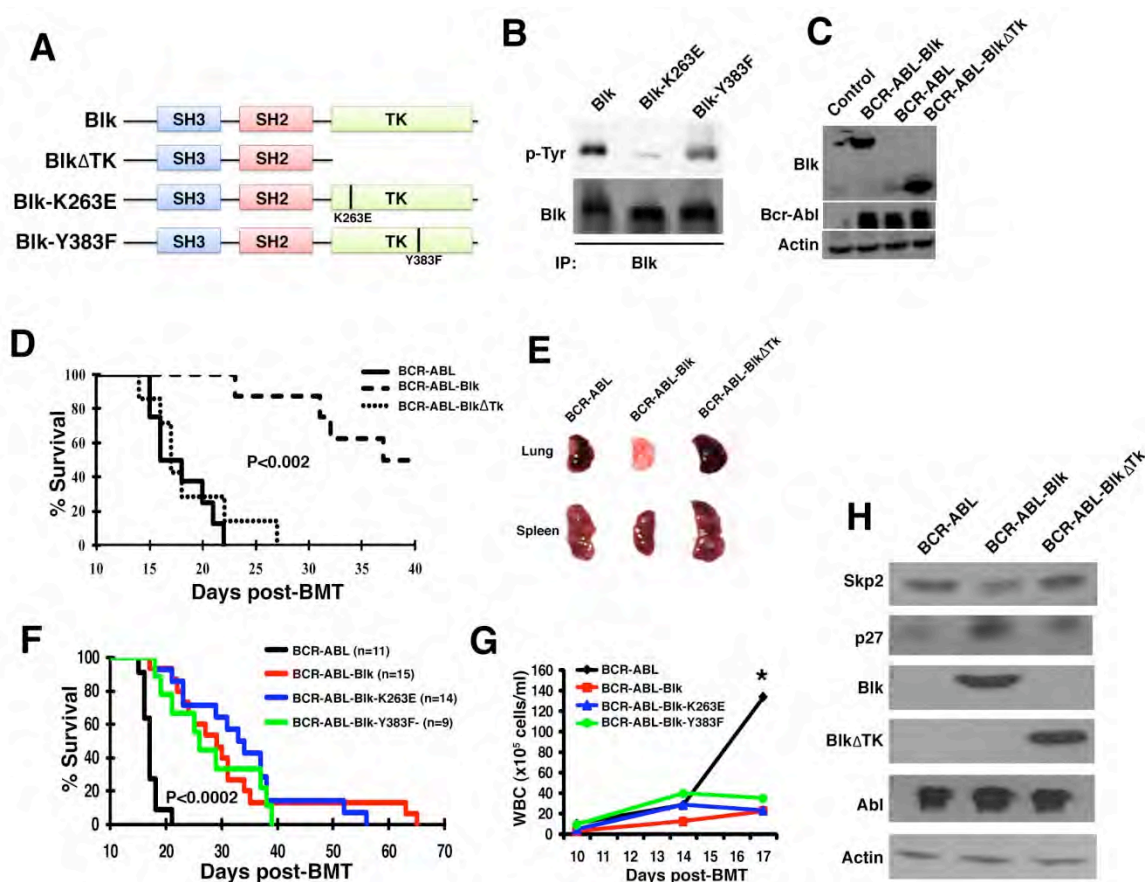
To determine whether Blk kinase activity was required for suppression of CML we analyzed three Blk mutants: deletion of the entire kinase domain ( $\Delta$ Tk), K263E, and Y383F (**Fig. 7A**). The K263E mutation causes a loss of Blk kinase activity, and the Y383F mutation reduces Blk autophosphorylation<sup>215</sup>. We cloned these three Blk mutants into the BCR-ABL retroviral construct. As expected, following transfection in 293T cells, all three Blk mutants were expressed (**Fig. 7B,C**), and kinase activity of Blk-K263E was almost completely lost and autophosphorylation of Blk-Y383F was significantly reduced (**Fig. 7B**). To test the effect of the three Blk mutants on BCR-ABL-induced CML development, we transduced bone marrow cells with BCR-ABL, BCR-ABL-Blk, BCR-ABL-Blk( $\Delta$ Tk), BCR-ABL-Blk-K263E, or BCR-ABL-Blk-Y383F, which had similar viral titers (**Fig. S8A, B**). The transduced bone marrow cells were transplanted into recipient mice to induce CML. We found that recipients of bone marrow cells transduced with BCR-ABL-Blk( $\Delta$ Tk) or BCR-ABL alone developed CML similarly, as shown by survival (**Fig. 7D**) and infiltration of leukemic cells into the lung and spleen (**Fig. 7E**). Thus, the kinase domain of Blk is required for suppression of CML development. Surprisingly, in recipients of BCR-ABL-Blk-K263E- and BCR-ABL-Blk-Y383F-transduced bone marrow cells CML development was also suppressed (**Fig. 7F**), which correlated with lower numbers of white blood cells (**Fig. 7G**). Thus, Blk suppresses CML development through a kinase-independent mechanism.

The results from several previous studies raised the possibility that the ability of Blk to stimulate p27 expression involves the S-phase kinase associated protein 2 (Skp2).

For example, p27 levels are inversely correlated with Skp2 expression<sup>216</sup>; BCR-ABL stimulates cell cycle progression by promoting Skp2-mediated degradation of p27<sup>211</sup>; and Skp2 is required for BCR-ABL induced myeloproliferative disease<sup>217</sup>. **Fig. 7H** shows that Blk prevented BCR-ABL-induced Skp2 expression, which was dependent on the Blk kinase domain.

**Figure 7. The inhibitory effect of Blk on CML does not require Blk kinase activity.**

(A) Schematic structures of Blk mutants. SH, Src-homology; TK, tyrosine kinase. (B) Western blot analysis monitoring the phosphorylation status of Blk-K263E, and the reduced phosphorylation level of Blk-Y383F, as compared to that of WT Blk. (C) Western blot analysis monitoring expression of Blk, Blk $\Delta$ Tk, and BCR-ABL in 293T cells. (D) Kaplan-Meier survival curves for recipients of BCR-ABL-, BCR-ABL-Blk-, or BCR-ABL-Blk $\Delta$ Tk-transduced bone marrow cells (n=8 for each group). (E) Gross appearance of the lungs and spleens of recipients of BCR-ABL-, BCR-ABL-Blk- or BCR-ABL-Blk $\Delta$ Tk-transduced bone marrow cells at 14 days after BMT. (F) Kaplan-Meier survival curves for recipients of BCR-ABL- (n=11), BCR-ABL-Blk- (n=15), BCR-ABL-Blk-K263E- (n=14) or BCR-ABL-Blk-Y383F- (n=9) transduced bone marrow cells. (G) The total number of white blood cells in peripheral blood of recipients of BCR-ABL-, BCR-ABL-Blk-, BCR-ABL-Blk-K263E- or BCR-ABL-Blk-Y383F-transduced bone marrow cells at days 10, 14 and 17 after BMT (\*P<0.006). (H) Western blot analysis indicated that Blk but not the truncated Blk $\Delta$ Tk, down-regulated Skp2 expression.



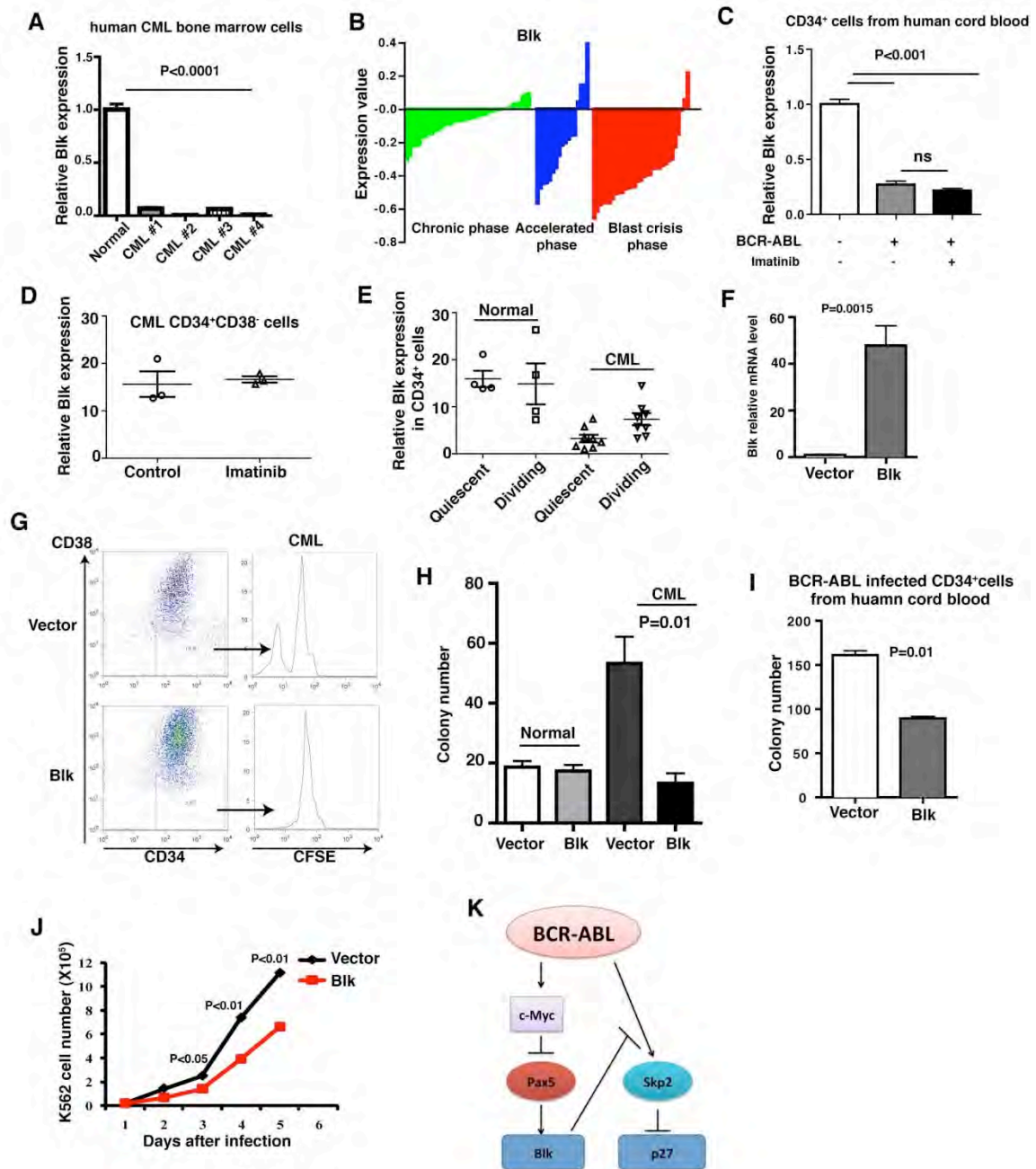
**Figure 7. The inhibitory effect of Blk on CML does not require Blk kinase activity.**

### Blk functions as tumor suppressor in human CML cells

The results described above prompted us to investigate whether Blk has a comparable tumor suppressor function in human CML cells. In the first series of experiments we asked whether *Blk* expression was lost in human cells expressing BCR-ABL and during CML development. **Fig. 8A** showed that *Blk* expression was substantially lower in bone marrow cells from human CML patients compared to normal human bone marrow cells. Consistent with our results, we analyzed a publicly available gene expression profiling database of human CML bulk CD34<sup>+</sup> cells<sup>218</sup>, and found that *Blk* expression was markedly down-regulated in the majority of CML patients in chronic phase, accelerated phase and blast crisis (**Fig. 8B**). Next, we transduced human cord blood CD34<sup>+</sup> cells with BCR-ABL and measured *Blk* expression. **Fig. 8C** showed that BCR-ABL significantly lowered *Blk* expression and this effect was not reversed by imatinib. Consistently, through analyzing another public microarray data of human CML CD34<sup>+</sup>CD38<sup>-</sup> cells<sup>219</sup>, we also found that *Blk* expression did not changed by imatinib treatment in CML CD34<sup>+</sup>CD38<sup>-</sup> cells (**Fig. 8D**). We further asked whether *Blk* expression in quiescent and dividing CD34<sup>+</sup> cells was downregulated in a public database of human CML quiescent stem/progenitor cells<sup>220</sup>. As expected, both quiescent and dividing CD34<sup>+</sup> cells from CML patients showed dramatically lower *Blk* expression than that in normal quiescent and dividing CD34<sup>+</sup> cells (**Fig. 8E**); interestingly, *Blk* expression was significantly lower in quiescent population than dividing cells from CML patients but not normal control (**Fig. 8E**). Together, the above data suggest that *Blk* might play tumor suppressive role in human CML stem cells.

In a second set of experiments, we tried to determine the functional effect of *Blk* on human CML stem/progenitor cells. To be able to infect quiescent cells, we used a lentivirus vector to express Blk (**Fig. 8F**). We found that Blk overexpression inhibited CML CD34<sup>+</sup>CD38<sup>-</sup> stem cells entry into cell cycle (**Fig. 8G**). We also performed a colony-formation assay, which provides an assessment of progenitor function<sup>221</sup>, and found that Blk overexpression inhibited the colony-forming ability of human CML but not normal bone marrow cells (**Fig. 8H**). We also found that Blk expression inhibited the colony-forming ability of BCR-ABL-transduced human cord blood CD34<sup>+</sup> cells (**Fig. 8I**). Furthermore, we found that overexpression of Blk induced the apoptosis of CML stem/progenitor cells (**Fig. S9**). To determine whether Blk inhibits proliferation of human CML cells, we transduced BCR-ABL<sup>+</sup> human K562 cells with a retrovirus co-expressing Blk and GFP or, as a control, GFP alone. **Fig. 8J** showed that Blk-expressing K562 cells grew significantly more slowly than cells that did not express Blk. Collectively, the results presented in **Figure 8** demonstrate that Blk functions as a tumor suppressor in human CML stem/progenitor cells.

**Figure 8 Blk functions as tumor suppressor in human CML cells.** (A) Real-time RT-PCR analysis was performed to detect *Blk* expression in bone marrow cells from CML patients and normal human bone marrow cells. ( $P < 0.0001$ ). (B) *Blk* expression in human CML samples. Microarray analysis of expression of *Blk* in bone marrow and peripheral blood CD34<sup>+</sup> cells from 42 chronic (green), 17 accelerated (blue) and 31 blast crisis phase (red) CML patients. (C) Real-time RT-PCR analysis monitoring *Blk* expression in BCR-ABL-transduced human CD34<sup>+</sup> cord blood progenitor cells. CD34<sup>+</sup> cells from human cord blood were transduced with *BCR-ABL* retrovirus. *Blk* expression was determined by RT-PCR analysis using sorted *BCR-ABL*-expressing CD34<sup>+</sup> cells. *BCR-ABL*-transduced CD34<sup>+</sup> cells were also treated by imatinib (1  $\mu$ M) for 24 hours. Imatinib treatment did not rescue the down-regulation of *Blk* expression. ( $P < 0.001$ ; ns, no significance. Mean values ( $\pm$  s.e.m) are shown. (D) *Blk* expression in human CML CD34<sup>+</sup>CD38<sup>-</sup> cells did not affect by imatinib treatment. ( $n=3$ ) (E) The expression of *Blk* in normal and CML quiescent and dividing stem/progenitor cells from public microarray. (F) Real-time RT-PCR analysis of *Blk* expression in primary chronic phase bone marrow cells from CML patients transduced with either an empty lentivirus (pLenti-puro) or virus expressing *Blk* (pLenti-*Blk*-puro). ( $P=0.0015$ ). (G) FACS analysis showed *Blk* inhibited CML CD34<sup>+</sup>CD38<sup>-</sup> cells proliferation. (H) *Blk* overexpression inhibited colony-forming ability of human CML bone marrow cells but not normal cells. Equal numbers of human CML bone marrow cells transduced with empty or *Blk* expressing lentivirus were plated in cytokine-supplemented methylcellulose in the presence of puromycin. ( $P=0.01$ ). (I) *Blk* overexpression inhibited colony-forming ability of BCR-ABL-expressing CD34<sup>+</sup> cells. CD34<sup>+</sup> cells from human cord blood were co-transduced BCR-ABL-GFP-expressing retrovirus and either an empty lentivirus or lentivirus expressing *Blk*, and colony-forming assay were performed in cytokine-supplemented methylcellulose in the presence of puromycin. ( $P=0.01$ ) (J) Growth curves of FACS sorted human K562 cells after transduction with retrovirus expressing *Blk-GFP* or *GFP* alone. (K) Molecular model of the *Blk* pathway in LSCs.



**Figure 8. Blk functions as tumor suppressor in human CML cells**



## Discussion

In this study we have shown that Blk functions as a tumor suppressor in CML through a pathway that is summarized in **Fig. 8K** and discussed below. Consistent with this tumor suppressor function, *Blk* expression is down-regulated by BCR-ABL in both mouse and human hematopoietic cells, and in bone marrow cells from CML patients. Of particular significance, expression profiling experiments indicate that *Blk* expression is markedly down-regulated in LSCs from the majority of CML patients in chronic phase, accelerated phase and blast crisis. Thus, suppression of *Blk* expression begins at an early stage of CML and is maintained throughout the course of disease.

Although imatinib and other BCR-ABL kinase inhibitors can induce a complete cytogenetic response in the majority of CML patients in chronic phase, they are incapable of eradicating LSCs<sup>61-63</sup>. We show that Blk markedly suppresses proliferation of LSCs without affecting normal HSCs or hematopoiesis. Thus, the Blk pathway identified here provides a selective target for eradicating LSCs and treating CML. For example, BCR-ABL down-regulates *Blk* expression to efficiently induce CML, and restoration of *Blk* expression or up-regulation of other genes in the Blk pathway, such as *Pax5* and *p27*, could be used to treat CML. Our results demonstrate the feasibility and effectiveness of specifically targeting cancer stem cells.

The strategy of selectively targeting LSCs contrasts sharply with other therapeutic approaches to inhibit the function of genes that have essential roles in both LSCs and normal HSCs<sup>91,92,110,222,223</sup>. For example, the Wnt signaling pathway has been shown to be critical in the regulation of hematopoietic stem and progenitor cell function<sup>222,223</sup>, and

deletion of the  $\beta$ -catenin gene causes a profound defect in LSCs and subsequent induction of CML by BCR-ABL<sup>91,224</sup>. In addition, inhibition of the hedgehog pathway impairs both LSCs and normal HSCs<sup>92,110</sup>.

The finding that Blk functions as a tumor suppressor role is somewhat unexpected because other Src family kinases have been found to promote leukemogenesis<sup>86,145,146,151</sup>. Paradoxically, Blk promotes normal B cell development by cooperating with other Src family members. Pax5, which we show functions upstream of Blk, is also required for normal B cell development<sup>225,226</sup>. Deletions and mutations of *Pax5* have been indentified in human acute lymphoid leukemia<sup>227,228</sup>, suggestive of a tumor suppressor function.

Pax5 mediates down-regulation of *Blk* by BCR-ABL through c-Myc. We also show that p27 mediates the inhibitory effect of *Blk* on LSCs, although it is likely that there are other downstream Blk target genes. Mechanistically, we show that Blk up-regulates *p27* through downregulation of *Skp2*. We have previously shown that LSCs in CML are also positively regulated by the *Alox5* gene, which is up-regulated by BCR-ABL<sup>85</sup>. Our unpublished data suggest that Blk regulates *Alox5* in LSCs, and it will be important to further elucidate the functional relationship between *Blk* and *Alox5* in LSCs.

We found that Blk and p27 do not suppress proliferation of normal HSCs. Consistent with our results, a previous study found that p27 has no effect on the number and self-renewal ability of HSCs<sup>229</sup>. Although a ten-fold overexpression of *Pax5* modestly inhibited normal HSCs (data not shown), we believe that restoration of *Pax5* expression to a normal endogenous level in HSCs would not significantly inhibit

proliferation. In contrast to the modest effect of Pax5 on HSCs, *Pax5* overexpression strongly inhibited proliferation of LSCs and CML development.

Blk is a tyrosine kinase but unexpectedly we found that Blk tumor suppressor function is not dependent upon its kinase activity. Thus, when a Src kinase inhibitor, such as dasatinib, is used to treat CML, it will not inhibit Blk tumor suppressor activity.

Down-regulation of *Blk* by BCR-ABL is not reversed following inhibition of BCR-ABL kinase activity, which is consistent with the inability of imatinib to kill LSCs.

Importantly, we show that Blk inhibits proliferation of human CML stem cells, providing a rationale for targeting LSCs by restoration of the Blk pathway.

### Material and methods

**Samples.** Cord blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. CD34<sup>+</sup> cells were enriched using the MACS CD34 progenitor kit (Miltenyi Biotec). Transduction of cord blood CD34<sup>+</sup> cells were performed as described previously<sup>230</sup>. Six human CML bone marrow samples contained greater than 97% (in average) of leukemia cells based on the karyotyping results for the t(9;22) translocation. This result is supported by the FISH analysis of the chimeric BCR-ABL oncogene in the cells. Human bone marrow CML cells were cultured in Iscoves Modified Dulbecco medium (Sigma) supplemented with a serum substitute (BIT; StemCell), 40µg/mL low-density lipoproteins, 100ng/mL recombinant human Flt3-ligand, 100ng/mL steel factor, 20ng/mL recombinant human interleukin-3 (IL-3), IL-6, and granulocyte-colony-stimulating factor<sup>231</sup>. For CML stem cell proliferation assay, sorted human CML CD34<sup>+</sup>

cells were transduced with vector or Blk expressing lentivirus, and labeled with 1 $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE), and cultured for 4 days in vitro.

**Mice.** *Blk*<sup>-/-</sup> mice were kindly provided by Dr. Alexander Tarakhovsky (Rockefeller University). *p27*<sup>-/-</sup>, C57BL/6J-CD45.1, C57BL/6J-CD45.2 mice were obtained from The Jackson Laboratory. All mice were in C57BL/6J background

**Cell culture.** K562 cells were obtained from ATCC, and maintained in RPMI 1640 plus 10% fetal bovine serum (FBS). 293T cells were cultured in DMEM median plus 10% FBS.

**Generation of retrovirus and lentivirus Stocks.** The retroviral constructs MSCV-IRES-GFP, MSCV-BCR-ABL-IRES-GFP, MSCV-Blk-IRES-GFP, MSCV-*BCR-ABL-IRES-Blk-IRES-GFP*, and MSCV-*BCR-ABL-IRES-Pax5-IRES-GFP* were used to generate high-titer, helper-free, replication-defective ecotropic virus stock by transient transfection of 293T cells as previously described<sup>23</sup>. Lentiviral vector (pLenti-Puro) was a kind gift from Dr. Eric Campaus (University of Massachusetts Medical School). Lentiviral particles were produced by cotransfection of 293T cells with pLP1, pLP2, VSV-G and empty vector or pLenti-hBlk-Puro. Lentiviral shRNA vector pLKO.1 were from OpenBiosystems. The targeted BCR-ABL sequences are as follows: sense 5'-ctgaccaactcgtgtgtgaaa-3', antisense 5'-tttcacacacgagttggtcag-3'.

**Bone marrow transduction/transplantation.** 8 to 12 week-old C57BL/6 mice were used for bone marrow transduction/transplantation. Retroviral transduction and transplantation of mouse bone marrow cells for inducing CML by BCR-ABL had been described previously<sup>23,86,232</sup>.

**Flow cytometry analysis.** For stem cell analysis, bone marrow cells were suspended in staining medium (Hank's Balanced Salt Solution (HBSS) with 2% heat-inactivated calf serum), and incubated with biotin-labeled lineage antibody cocktail containing a mixture of antibodies against CD3, CD4, CD8, B220, Gr-1, Mac-1 and Ter119. After washing, the fluorochrome-labeled secondary antibody (APC-Cy7-conjugated Streptavidin) for recognizing biotin and PE-conjugated c-Kit and APC-conjugated Sca-1 antibodies were added to the cells. Long-term and short-term LSCs were distinguished by the CD34 antibody. LSCs were analyzed by FACS. All these antibodies were purchased from eBioscience.

**Leukemia stem cell culture.** For mouse leukemia stem cell culture, bone marrow cells isolated from CML mice were cultured in vitro in the presence of stemspan SFEM, SCF, IGF-2, TPO, heparin, and  $\alpha$ -FGF as reported previously for culturing hematopoietic stem cells<sup>232</sup>.

**In Vitro methylcellulose colony formation assay.** Human CML cells or BCR-ABL transformed human cord blood CD34<sup>+</sup> cells were transduced with vector or Blk lentivirus and cultured in methylcellulose medium containing 2.5 $\mu$ g/mL puromycin for selection (Methocult GF H4435; Stem Cell Technologies). Colonies were counted under microscope after 7 days.

**Chromatin immunoprecipitation.** ChIP assays were performed. Briefly, 3x10<sup>7</sup> ENU or BaF3 cells were incubated with 1% formaldehyde for 10 min at room temperature before crosslinking was quenched by addition of 0.125 M glycine. Cells were collected by centrifugation and lysed in lysis buffer containing 50mM Tris-HCl (pH 8.0), 10 mM

EDTA, 0.5% SDS, proteinase inhibitors and phosphatase inhibitors. The cells suspension was sonicated seven times for 10s each with 2-min intervals on ice using a Misonix Sonicator 3000 at output 8. Sonicated chromatin was then incubated at 4°C overnight with 5 µg of the appropriate antibody:  $\alpha$ -c-Myc (Santa Cruz),  $\alpha$ -EBF1 (Avaon).

Immunoprecipitated DNA was amplified by real-time PCR using the following primers: myc-chip-for (5'-gacgtgtctggggacatcttg-3'), myc-chip-rev (5'-gtccgaaggcaccgtgaaatg), EBF1-chip-for (5'-actacggagactacttggtgg), and EBF1-chip-rev (5'-caccggacatcgcaaattcac)

**Luciferase reporter assays.**  $2 \times 10^5$  NIH3T3 cells were seeded in six-well plates 24h before transfection. 1 µg of the Pax5 promoter luciferase reporter plasmid (pGL3-Pax5), kindly provided by Dr. Kathryn Calame (Columbia University), was cotransfected with various amounts of EBF1, IRF8, c-Myc, or control vectors, into NIH3T3 cells by the calcium phosphate method. 48h after transfection, cells were harvested and luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega).

**DNA microarray and data analysis.** Bone marrow cells were isolated from CML mice 14 days after the induction of the disease. *BCR-ABL*-expressing or *BCR-ABL/Blk*-expressing (transduced with the *BCR-ABL-GFP* or *BCR-ABL-Blk-GFP*, respectively)  $GFP^+Lin^-c-Kit^+Sca-1^+$  cells (representing LSCs) were stored by FACS directly into RNeasy Lysis Buffer (Qiagen) and homogenized in RLT Buffer (RNeasy Micro Kit) (Qiagen). Total RNA was isolated by following the protocol for the RNeasy Micro Kit. RNA was amplified, labeled, and approximately 2.0 µg of fragmented and biotin-labeled cDNA was then hybridized onto Mouse Genome 430 2.0 microarray (Affymetrix). Relative fold

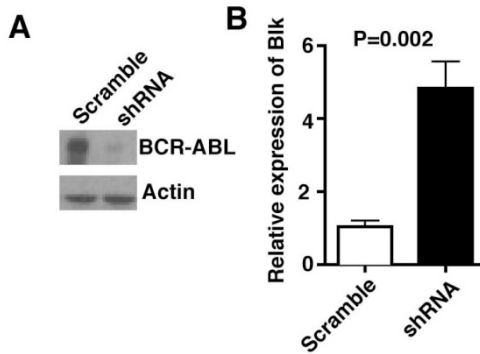
change (RFC) of a gene between *BCR-ABL*-expressing or *BCR-ABL/Blk*-expressing LSCs was calculated using the formula:  $\text{RFC} = (\text{sign}(D) + (D == 0)) * 2^{\text{abs}(D)}$ . The log2 of the RFC value for the gene was shown. The detailed analysis of the microarray experiment and data were described in the Supplemental Information and the microarray data was deposited into GEO database (GSE36096). The expression of Blk gene in human CML cells, quiescent and dividing CML CD34<sup>+</sup> cells, and CD34<sup>+</sup>CD38<sup>-</sup> CML stem cells treated with imatinib were analyzed independently in publicly available microarray data sets including GSE4170 for human CML bulk CD34<sup>+</sup> cells, GSE24739 for quiescent (G0) and dividing (G1) CML leukemia stem cells, and GSE20876 for CML CD34<sup>+</sup>CD38<sup>-</sup> leukemia stem cells with/without imatinib treatment. The probe-level raw intensity data were normalized and summarized into probes-set level data using Probe Logarithmic Intensity Error (PLIER) method. The BLK gene expression were extracted and further re-normalized at probe-set level by a set of suitable reference genes, the significance of changes between relevant groups was assessed by moderated t-test.

**Immunoprecipitation, western blotting and antibodies.** Protein lysates were prepared by lysing cells in RIPA buffer containing 25mM TrisHCl, 150mM NaCl, 1%NP-40, 1% sodium deoxycholate, 0.1% SDS. Blk was immunoprecipitated with anti-Blk antibody and blotted with anti-phospho-tyrosine (p-Tyr) antibody. Antibodies against c-Abl, Blk, p-Tyr, Pax5, p27, p21, CDK2, Skp2, c-Myc and beta-actin were purchased from Santa Cruz Biotechnology.

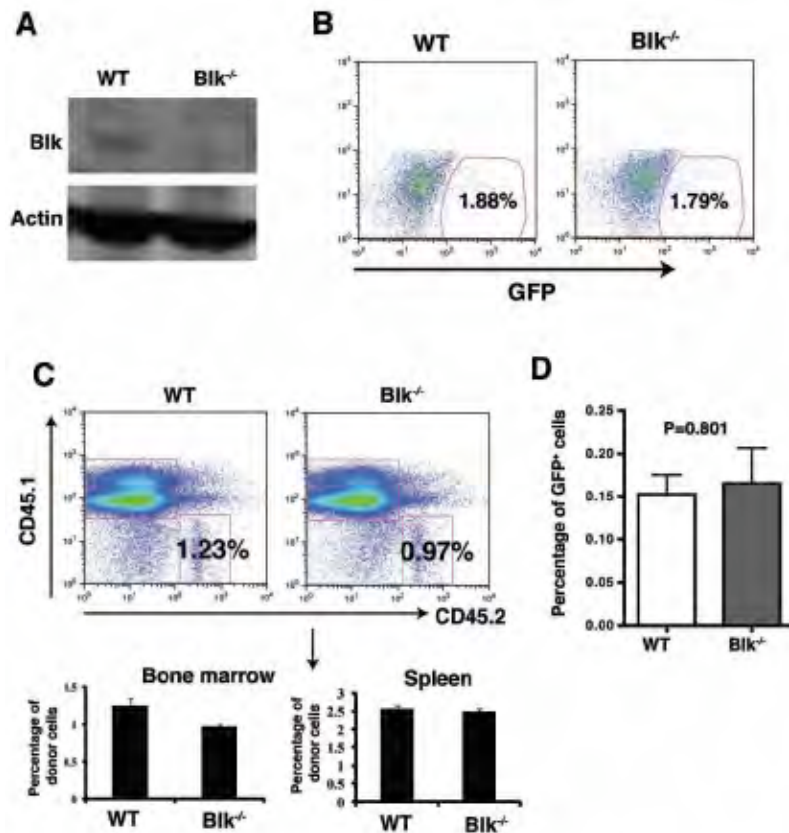
**Real time-PCR.** Total RNA was isolated from GFP<sup>+</sup>LK<sup>+</sup>S<sup>+</sup> bone marrow cells from mice using the RNeasy Mini kit (Qiagen). cDNA was synthesized using the Ovation-Pico cDNA synthesis method. All real time PCR reactions were done using the Applied Biosystems 7500. 25μL reaction system was composed of 12.5μL SYBR Green, 2.5μL 20uM primer mixture, 10ng cDNA and nuclease-free water. All experiments were performed in triplicate. β-actin was the internal control. The following are specific primer sequences: *Pax5* sense: gtacagcagcccccaatc; antisense: accttcacccctcttgcgttt; *Blk* sense: tgtgtgcaccagagagccattta; antisense: tgtcaatctgccttgaaggaca; *EBF1* sense: tgcagatctggttgaagccctgta; antisense: atccctgcatggaccgaagtgtta; *Skp2* sense: aaacctttgtgggtggctctg; antisense: cttaagtcgaggcgatgag; *BCR* sense: cgtgtgtgaaactccagactgtca; *ABL* antisense: cttcagcggccagtagcatct; *Actin* sense: atggaatcctgtggcatcca; antisense: cgctcaggaggagcaatgat.

**Statistical analysis.** Results are given as mean ± s.e.m. Statistical analysis was performed by Students' t test for all column statistis. For survival curves, p values were obtained using a Log-rank test.



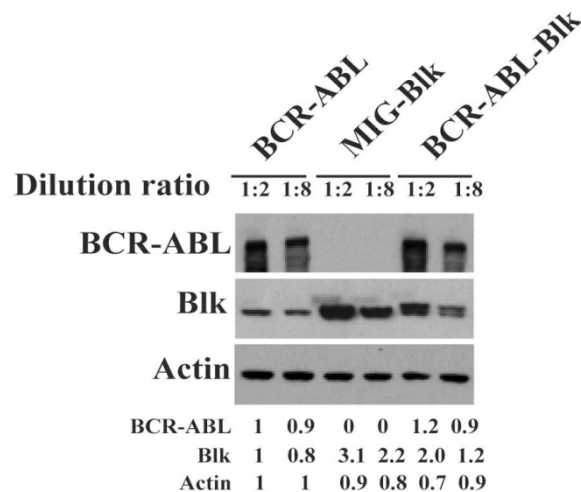


**Figure S1. Knockdown BCR-ABL expression restores Blk expression.** (A) Western blot analysis showed marked decrease of BCR-ABL expression after lentivirus shRNA infection. (B) Real time RT-PCR analysis showed increased Blk expression in leukemia cells after knockdown of BCR-ABL.

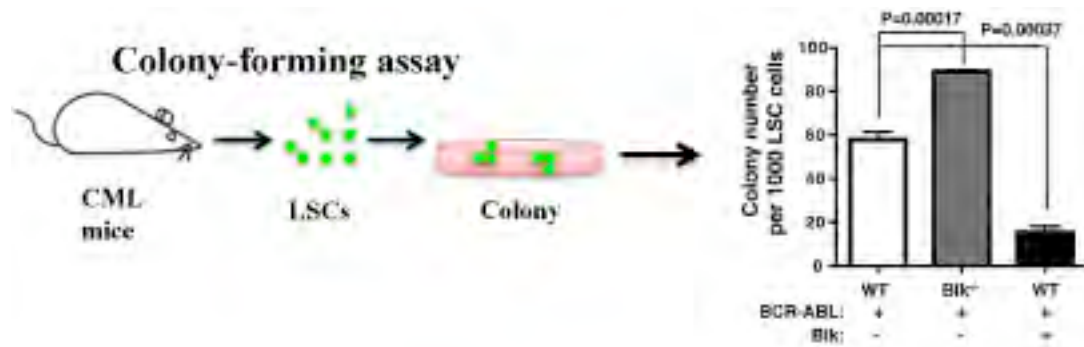


**Figure S2. Blk deletion does not affect the transfection efficiency and homing ability of bone marrow cells.** (A) Detection of Blk Expression in Bone Marrow Cells of WT and Blk<sup>-/-</sup> Mice by western blotting. (B) Blk does not affect the retroviral transduction efficiency of bone marrow cells. Bone marrow cells from 5-FU treated WT or Blk<sup>-/-</sup> mice

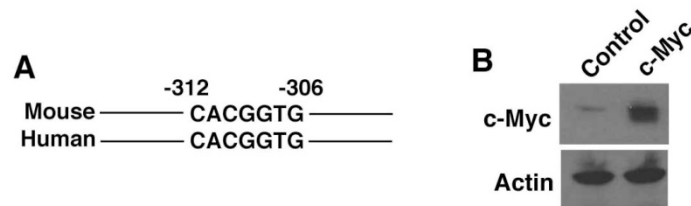
were transduced with BCR-ABL-GFP, and the percentages of GFP<sup>+</sup> cells before BMT were analyzed by FACS. (C) Homing assay of WT or *Blk*<sup>-/-</sup> bone marrow cells.  $6 \times 10^6$  WT or *Blk*<sup>-/-</sup> bone marrow cells (CD45.2) were transplanted into lethally irradiated recipients (CD45.1) and the donor-derived bone marrow cells (CD45.2) were detected by FACS in 3 hours after BMT. Mean values ( $\pm$  SEM) are shown. (D) WT or *Blk*<sup>-/-</sup> bone marrow cells transduced with BCR-ABL retrovirus were cultured for 6 days. Equal number of GFP<sup>+</sup> cells were transplanted into each lethally irradiated recipient mice, and the percentages of GFP<sup>+</sup> cells in bone marrow of recipients were analyzed 3 hours after BMT (n=4). Mean values ( $\pm$  SEM) are shown.



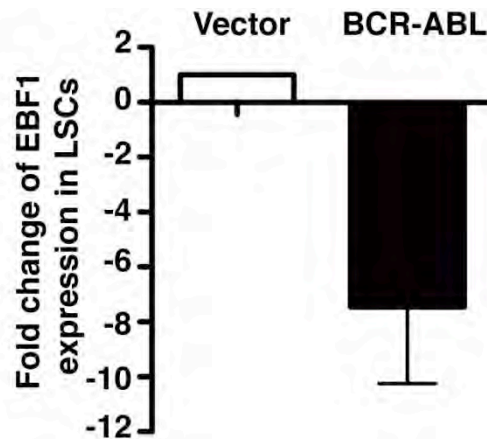
**Figure S3. Functional testing of *BCR-ABL* and *Blk* Retroviruses in 3T3 Cells.** 3T3 cells were transduced with retrovirus (1:2 or 1:8 dilution) expressing BCR-ABL-GFP, Blk-GFP, or BCR-ABL-Blk. Expression of BCR-ABL and Blk proteins were detected by Western blotting



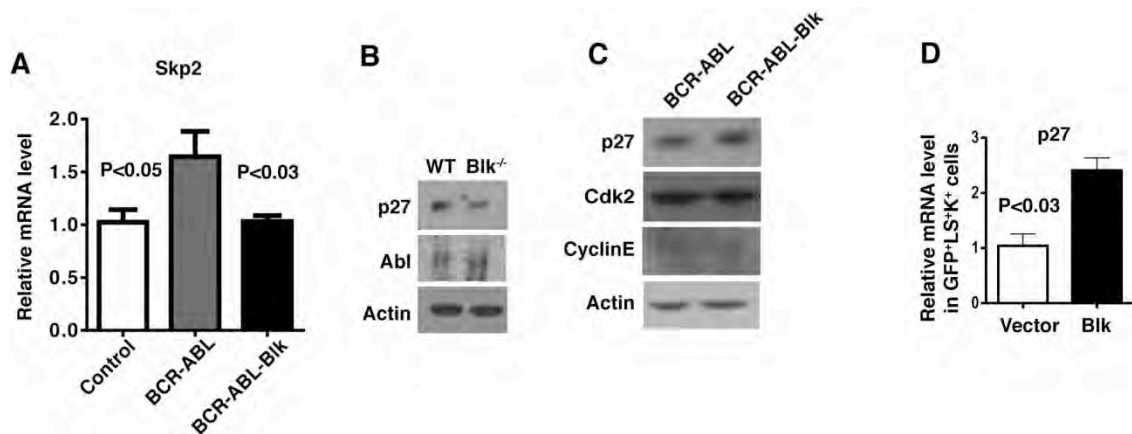
**Figure S4. Blk suppress colony forming ability of LSCs.** LSCs from CML mice which received BCR-ABL transduced WT and Blk<sup>-/-</sup> boe marrow cells or received BCR-ABL-Blk transduced WT bone marrow cells were plated in methycellulose medium for colony culture. Compared with WT LSCs, Blk<sup>-/-</sup> LSCs generated more colonies, and Blk overexpression caused less colonies.



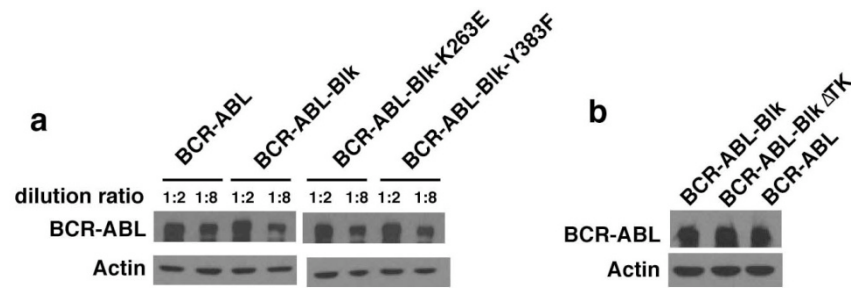
**Figure S5. The c-myc binding site in the Pax5 promoter.** (A) The sequence of c-Myc binding site in the Pax5 promoter is completely conserved in mouse and human. (B) Western blot analysis showed c-Myc expression. Pax5 overexpression has a modest effect on normal HSCs and Pax5 promoter has c-myc binding site.



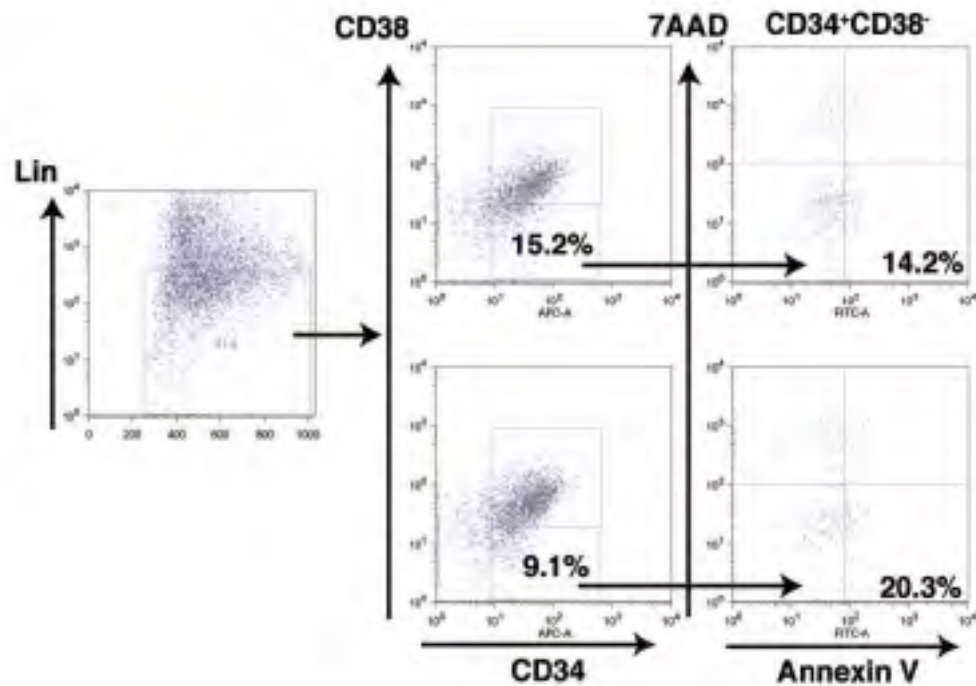
**Figure S6. EBF1 expression was down-regulated in BCR-ABL expressing LSCs.** Microarray analysis showing EBF1 expression in vector- and BCR-ABL-transduced LSCs. Mean values ( $\pm$  s.e.m) are shown.  $p < 0.05$ .



**Figure S7. Blk affects the expression of Skp2 and p27.** (A) Real time RT-PCR analysis showed Blk inhibited the induction of Skp2 expression by BCR-ABL. (B) p27 was down-regulated in myeloid leukemia cells. Myeloid leukemia cells were sorted from the spleen of CML mice receiving BCR-ABL transduced WT or *Blk*<sup>-/-</sup> bone marrow cells two weeks after CML induction, and p27 levels in the cells were detected by Western blotting. (C) Western blot analysis showed that Blk induced expression of p27 and p53 expression but not CDK2 and cyclin E. in bone marrow cells from CML mice which were cultured for 4 days in vitro under the stem cell culture conditions. (D) Real-time RT-PCR data showed a significant upregulation of *p27* in sorted Blk-expressing LSK cells. Mean values ( $\pm$  SEM) are shown ( $P < 0.01$ ).



**Figure S8. Normalization of the retrovirus titers of BCR-ABL, BCR-ABL-Blk, BCR-ABL-Blk-K263E, BCR-ABL-Blk-Y383F, and BCR-ABL-Blk $\Delta$ TK.** (A and B) NIH3T3 cells were infected with retroviruses, and 48h later, the levels of BCR-ABL proteins were analyzed by western blotting.



**Figure S9. Blk expression induces apoptosis of CML stem cells.** Lin<sup>-</sup>CD34<sup>+</sup> cells from CML patients were sorted and infected with Blk lentivirus. After puromycin selection, FACS analysis showed increased apoptosis in the presence of Blk.

**Table S1. List of up-regulated genes ( $P \leq 0.05$ )**

Symbol	Gene name	Relative fold change
<i>Igkv19-93</i>	immunoglobulin kappa chain variable 19-93	2.89
<i>Myo1h</i>	myosin 1H	2.1
<i>Ufsp1</i>	UFM1-specific peptidase 1	2.07
<i>Them4</i>	thioesterase superfamily member 4	1.92
<i>Igl-V2</i>	Immunoglobulin lambda chain, variable 2	1.89
<i>Clec9a</i>	C-type lectin domain family 9, member a	1.88
<i>Cldn22</i>	claudin 22	1.78
<i>Rab1b</i>	RAB1B, member RAS oncogene family	1.78
<i>Gnrh1</i>	gonadotropin releasing hormone 1	1.77
<i>Tsga14</i>	testis specific gene A14	1.75
<i>Plekho2</i>	pleckstrin homology domain containing, family O member 2	1.73
<i>Hist1h2ak</i>	histone cluster 1, H2ak	1.73
<i>Cyb5d1</i>	cytochrome b5 domain containing 1	1.72
<i>Prl7c1</i>	prolactin family 7, subfamily c, member 1	1.7
<i>Zfp459</i>	zinc finger protein 459	1.66
<i>Kcnrg</i>	potassium channel regulator	1.65
<i>Cmtm3</i>	CKLF-like MARVEL transmembrane domain containing 3	1.65
<i>Gcet2</i>	germinal center expressed transcript 2	1.62
<i>Olfir43</i>	olfactory receptor 43	1.62
<i>Rfc5</i>	replication factor C (activator 1) 5	1.6

**Table S2 List of down-regulated genes ( $P \leq 0.05$ )**

Symbol	Gene name	Relative fold change
<i>Khdc1a</i>	KH homology domain containing 1A	4.40
<i>Il1f9</i>	Interleukin 1 family, member 9	2.78
<i>Ublcp1</i>	ubiquitin-like domain containing CTD phosphatase 1	2.48
<i>Lcn2</i>	lipocalin 2	2.39
<i>Scrn3</i>	secernin 3	2.26
<i>Fabp4</i>	fatty acid binding protein 4, adipocyte	2.21
<i>Trem3</i>	triggering receptor expressed on myeloid cells 3	2.17
<i>Chi3l3</i>	chitinase 3-like 3	2.17
<i>Dhrs7</i>	dehydrogenase/reductase (SDR family) member 7	2.16
<i>Gldc</i>	glycine decarboxylase	2.10
<i>S100a5</i>	S100 calcium binding protein A5	2.08
<i>Ifitm6</i>	interferon induced transmembrane protein 6	2.06
<i>Clec4d</i>	C-type lectin domain family 4, member d	2.03
<i>Serpina3f</i>	serine (or cysteine) peptidase inhibitor, clade A, member 3F	1.97
<i>Nrg4</i>	Neuregulin 4	1.95
<i>Hdc</i>	histidine decarboxylase	1.94
<i>Bank1</i>	B-cell scaffold protein with ankyrin repeats 1	1.89
<i>Mt1</i>	metallothionein 1	1.84
<i>Il6</i>	Interleukin 6	1.83
<i>Gem</i>	GTP binding protein (gene overexpressed in skeletal muscle)	1.81

## CHAPTER IV

### **Scd1 plays a tumor suppressive role in survival of leukemia stem cells and the development of chronic myeloid leukemia**

The work described in the chapter has been published (Zhang et al., Mol. Cell. Biol. 2012 32:1776-1787)

#### **Abstract**

Chronic myeloid leukemia (CML) is derived from a stem cell and it is widely accepted that the existence of leukemia stem cells (LSCs) is one of the major reasons for the relapse of CML treated with kinase inhibitors. Key to eradicating LSCs is to identify genes that play a critical role in survival regulation of these stem cells. Using BCR-ABL-induced CML mouse model, here we show that expression of the stearoyl-CoA desaturase 1 (*Scd1*) gene is down-regulated in LSCs and that Scd1 plays a tumor suppressive role in LSCs with no effect on the function of normal hematopoietic stem cells (HSCs). Deletion of Scd1 causes acceleration of CML development and conversely overexpression of Scd1 delays CML development. In addition, using genetic approaches, we show that Pten, p53 and Bcl2 are regulated by Scd1 in LSCs. Furthermore, we find that induction of Scd1 expression by a PPAR $\gamma$  agonist suppresses LSCs and delays CML development. Our results demonstrate a critical role for Scd1 in functional regulation of LSCs, providing a new anti-LSC strategy through enhancing Scd1 activity.



## Introduction

Leukemia stem cells (LSCs) are defined as transformed hematopoietic stem cell or progenitors that have acquired unlimited self-renewal ability. It is generally accepted that eradication of LSCs is required for effectively treating and eventually curing the diseases. Chronic myeloid leukemia (CML) is believed to originate from a hematopoietic stem cell (HSC) harboring Philadelphia chromosome formed through a reciprocal translocation between chromosomes 9 and 22. Human CML is induced by the BCR-ABL oncogene that produces the chimeric BCR-ABL protein functioning as a constitutively activated tyrosine kinase in leukemogenesis<sup>1,2,6</sup>. Although BCR-ABL kinase inhibitors are highly effective in treating chronic phase CML patients<sup>39</sup>, they do not completely eradicate LSCs<sup>39,62,63,201</sup>. Therefore, there is an urgent need for developing anti-LSC strategies, and this approach relies on identification of critical target genes in LSCs.

Accumulating evidence shows a close relationship between cancer and metabolism<sup>233</sup>. Cancer cells display changes in fatty acid metabolism<sup>234</sup>, which is associated with modulations of expression and activity of lipogenic enzymes. One such an enzyme is stearoyl-CoA desaturase (Scd). Scd is an endoplasmic reticulum enzyme, belonging to a family of  $\Delta^9$ -fatty acid desaturase isoforms. Scd1 catalyzes the biosynthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA), which are the most abundant fatty acids present in mammalian organisms<sup>157</sup>. Scd1 expression is detected in almost all tissues with a predominant expression in liver, and is involved in regulating metabolic pathways related to preadipocyte differentiation, insulin sensitivity, metabolism, and tumorigenesis<sup>157,164-167</sup>. A recent study indicates that

inhibition of *Scd1* impairs lung cancer cell proliferation, survival and invasiveness<sup>165</sup>, suggesting that *Scd1* plays a supporting role in lung cancer cells. Another study shows that the level of *Scd1* expression correlates with predisposition to liver carcinogenesis<sup>166</sup>. However, the role of *Scd1* in leukemogenesis and hematopoiesis remains unknown, although it is suggested that fatty acid metabolism plays a role in leukemogenesis and hematopoiesis<sup>170,171</sup>. In this study, using BCR-ABL induced chronic myeloid leukemia mouse model, we found that expression of the *Scd1* gene was down-regulated in LSCs and deletion of *Scd1* accelerated CML development through affecting the function of LSCs but not normal HSCs, suggesting that *Scd1* plays a tumor suppressive role in BCR-ABL leukemogenesis. Our study provides a new strategy for targeting LSCs by enhancing the *Scd1* function.

## Results

### ***Scd1* plays a tumor suppressive role in CML development**

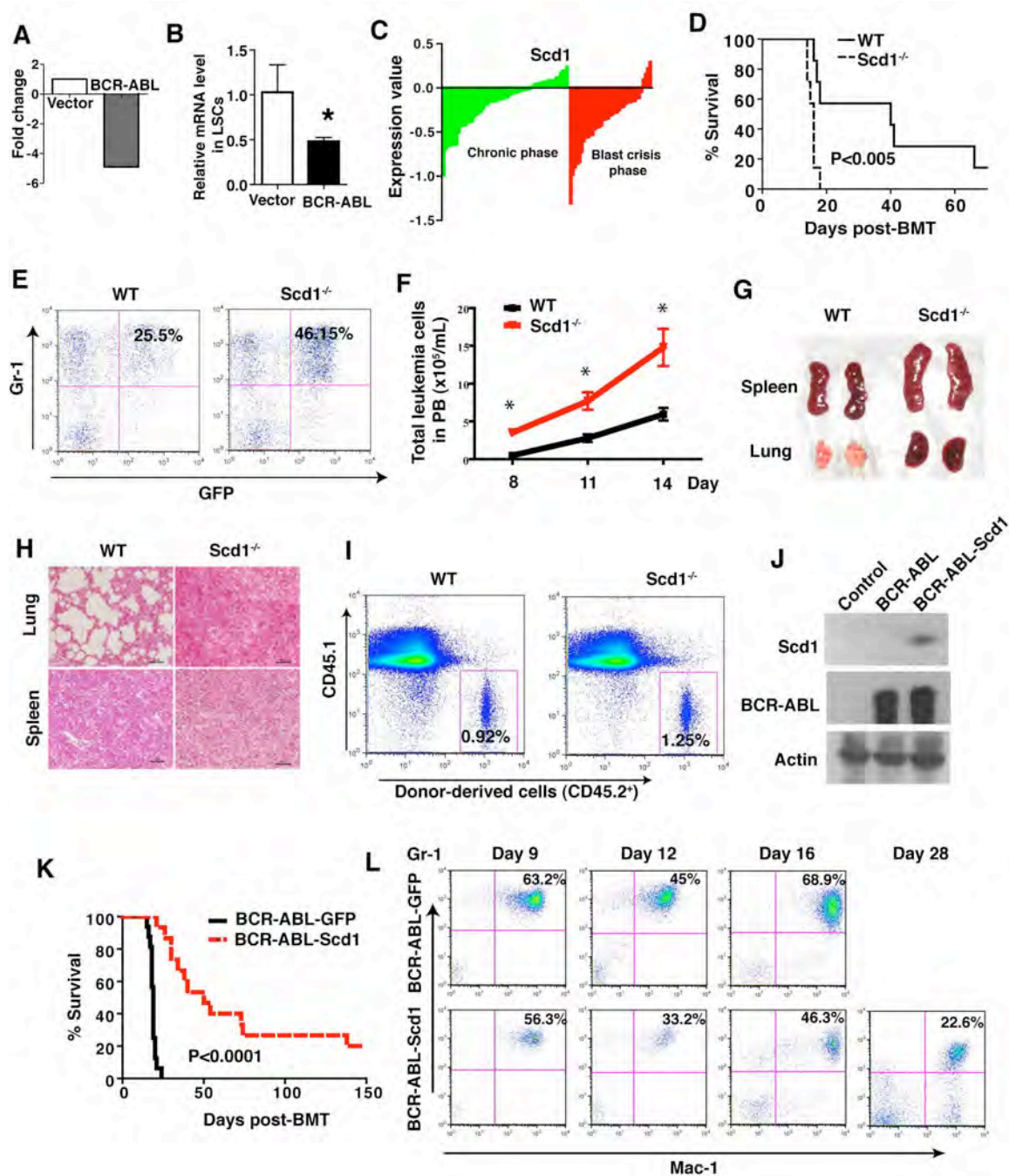
BCR-ABL-expressing HSCs (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells) in mice function as LSCs<sup>63</sup>. We analyzed the results from our previously published DNA microarray study (GEO submission: GSE10912), in which we compared the gene expression profiles between LSCs and normal HSCs. We found that expression of the *Scd1* gene was dramatically lower in BCR-ABL-expressing LSCs as compared to normal HSCs (**Fig. 1A**), and this down-regulation of *Scd1* in LSCs was confirmed by real time RT-PCR (**Fig. 1B**). Furthermore, we analyzed a publicly available gene expression profiling database of human CML stem cells<sup>218</sup>, and found that *Scd1* expression was markedly down-regulated

in the majority of CML patients in chronic phase and blast crisis (**Fig. 1C**). These results suggest that *Scd1* plays a tumor suppressive role in CML development. To test this idea, we first investigated whether *Scd1* deficiency causes acceleration of CML development using *Scd1* homozygous knockout (*Scd1*<sup>-/-</sup>) mice. We transduced bone marrow cells from 5-FU-treated wild type (WT) or *Scd1*<sup>-/-</sup> mice with the *BCR-ABL-GFP* retrovirus, followed by transplanting the transduced cells into lethally irradiated recipient mice to induce CML. We found that recipients of *BCR-ABL*-transduced *Scd1*<sup>-/-</sup> bone marrow cells developed and died of CML significantly faster than did recipients of *BCR-ABL*-transduced WT bone marrow cells (**Fig. 1D**). The accelerated CML development in the absence of *Scd1* correlated with a higher percentage and total numbers of GFP<sup>+</sup>Gr-1<sup>+</sup> myeloid leukemia cells in peripheral blood (**Fig. 1E, F**), and with more severe splenomegaly and lung hemorrhage (**Fig. 1G**). Histological examination showed more extensive infiltration of leukemia cells in the lung and spleen of CML mice receiving *BCR-ABL*-transduced *Scd1*<sup>-/-</sup> bone marrow cells (**Fig. 1H**). Because increased homing of donor bone marrow cells could lead to accelerated CML development, we compared the homing ability of *Scd1*<sup>-/-</sup> bone marrow cells with WT bone marrow cells 3 hours after the transplantation of the cells into recipient mice. We found that the percentages of donor-derived WT and *Scd1*<sup>-/-</sup> bone marrow cells in recipient mice were similar (**Fig. 1I**), indicating that *Scd1* deficiency does not significantly alter the homing ability of bone marrow cells.

Reversely, we overexpressed *Scd1* in donor bone marrow cells by transducing the cells with retrovirus expressing both *BCR-ABL* and *Scd1* (**Fig. 1J**) to examine whether

enforced expression of Scd1 causes a delay of CML development. We did observe that Scd1 overexpression caused a significant delay of CML development in secondary recipients of bone marrow cells from the primary CML mice (**Fig. 1K**). This delayed CML development correlated with lower percentages of myeloid cells in peripheral blood of the mice (**Fig. 1L**). Together, these results indicate that Scd1 plays an inhibitory role in CML development.

**Figure 1. *Scd1* plays a tumor suppressive role in CML development.** (A) Microarray analysis showed that *Scd1* expression was down-regulated in BCR-ABL-expressing LSCs as compared to normal stem cells that did not express BCR-ABL. (B) Real time RT-PCR analysis showed that *Scd1* expression was down-regulated in LSCs. The expression data were normalized to  $\beta$ -actin expression and shown as mean $\pm$ s.e.m. (n=3; \*, P<0.05). (C) *Scd* expression in human CML samples. Microarray analysis showed expression of *Scd1* in CD34<sup>+</sup> bone marrow and peripheral blood (PB) cells from 42 chronic (green), and 31 blast crisis phase (red) CML patients. (D) Kaplan-Meier survival curve for recipients of BCR-ABL-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells (n=7-8 mice per group; p<0.005). (E) The percentage of GFP<sup>+</sup>Gr-1<sup>+</sup> cells in peripheral blood of recipients of BCR-ABL-transduced bone marrow cells from WT or *Scd1*<sup>-/-</sup> donor mice at day 14 after bone marrow transplantation (BMT). (F) Total number of GFP<sup>+</sup>Gr-1<sup>+</sup> leukemia cells in PB of recipients of BCR-ABL-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells. (G) Gross appearance of the lungs and spleens from mice receiving BCR-ABL-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells at day 14 after BMT. (H) Hematoxylin/eosin staining of tissue sections from the lungs and spleens of mice receiving BCR-ABL-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells at day 14 after BMT (Scale bar: 50  $\mu$ m). (I) The loss of *Scd1* does not affect the homing ability of bone marrow cells.  $6 \times 10^6$  bone marrow cells from WT or *Scd1*<sup>-/-</sup> mice (CD45.2) were transplanted into lethally irradiated recipient mice (CD45.1). The donor-derived bone marrow cells (CD45.2) were detected by FACS in 3 hours after BMT. (J) Western blot analysis showed expression of *Scd1* and BCR-ABL in 293T cells transfected with BCR-ABL-*Scd1*. (K) Kaplan-Meier survival curve for secondary recipients of equal number of bone marrow cells from primary CML mice receiving BCR-ABL- or BCR-ABL-*Scd1*-transduced WT bone marrow cells. (n=14-16 mice per group; p<0.0001). (L) The percentages of myeloid cells (Gr-1<sup>+</sup>Mac-1<sup>+</sup>) in peripheral blood of secondary recipients of bone marrow cells from primary CML mice receiving BCR-ABL- or BCR-ABL-*Scd1*-transduced WT bone marrow cells at day 9, 12, 16, and 28 after BMT.



**Figure 1. Scd1 plays a tumor suppressive role in CML development.**

### **Scd1 deletion does affect the development of BCR-ABL induced B-ALL**

We also tested whether Scd1 is required for BCR-ABL induced B-ALL. We first assessed Scd expression in human B-ALL using a publicly microarray dataset (GSE5314) of 37 BCR-ABL-positive (21 of p190<sup>BCR-ABL</sup> and 16 of p210<sup>BCR-ABL</sup>) and 17 BCR-ABL-negative adult ALL<sup>235</sup>. We did not observe significant difference in Scd expression between BCR-ABL-positive ALL and BCR-ABL-negative ALL and between p190<sup>BCR-ABL</sup>-positive and p210<sup>BCR-ABL</sup>-positive ALL (**Fig. 2A**). These data suggest that Scd expression may not be relevant to BCR-ABL in ALL. Consistent with this idea, recipients of WT or *Scd1*<sup>-/-</sup> bone marrow cells transduced with *BCR-ABL* retrovirus developed and died of ALL with similar disease latency and survival (**Fig. 2B**), correlating with similar numbers and percentages of GFP<sup>+</sup>B220<sup>+</sup> leukemia cells in peripheral blood of the two groups of B-ALL mice (**Fig. 2C, D**). These results indicate that Scd1 is dispensable for BCR-ABL induced B-ALL.

**Figure 2. *Scd1* does not suppress B-ALL induced by BCR-ABL.** (A) Microarray analysis showing *Scd* expression in human B-ALL samples from 37 BCR-ABL-positive [21 of p190<sup>BCR-ABL</sup> (green) and 16 of p210<sup>BCR-ABL</sup> (red)] and 17 BCR-ABL-negative (blue) adult patients. (B) Kaplan-Meier survival curve for recipients receiving *BCR-ABL*-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells. Both groups of mice developed and died of ALL with similar disease latency (n=8 mice per group). (C) The total number of GFP<sup>+</sup>B220<sup>+</sup> cells in PB of recipients of *BCR-ABL*-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells. (D) FACS analysis showed no difference in the percentages of GFP<sup>+</sup>B220<sup>+</sup> cells in PB of recipients of *BCR-ABL*-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells.



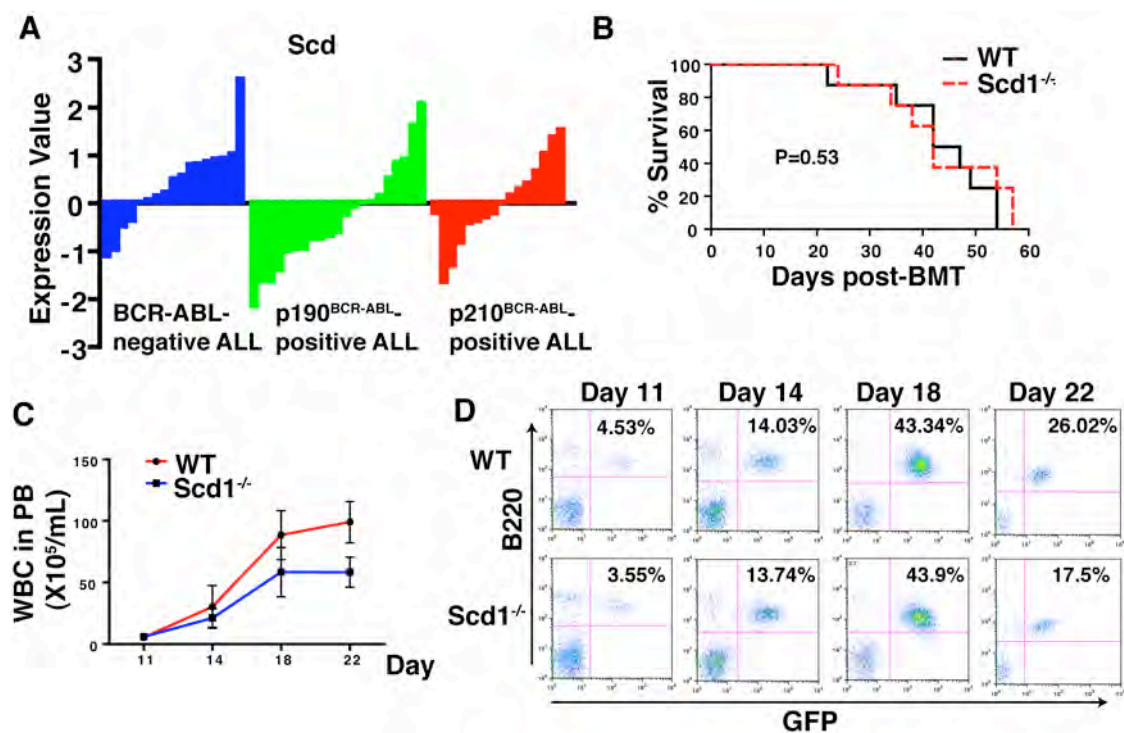


Figure 2. *Scd1* does not suppress B-ALL induced by BCR-ABL

### Scd1 suppresses LSCs

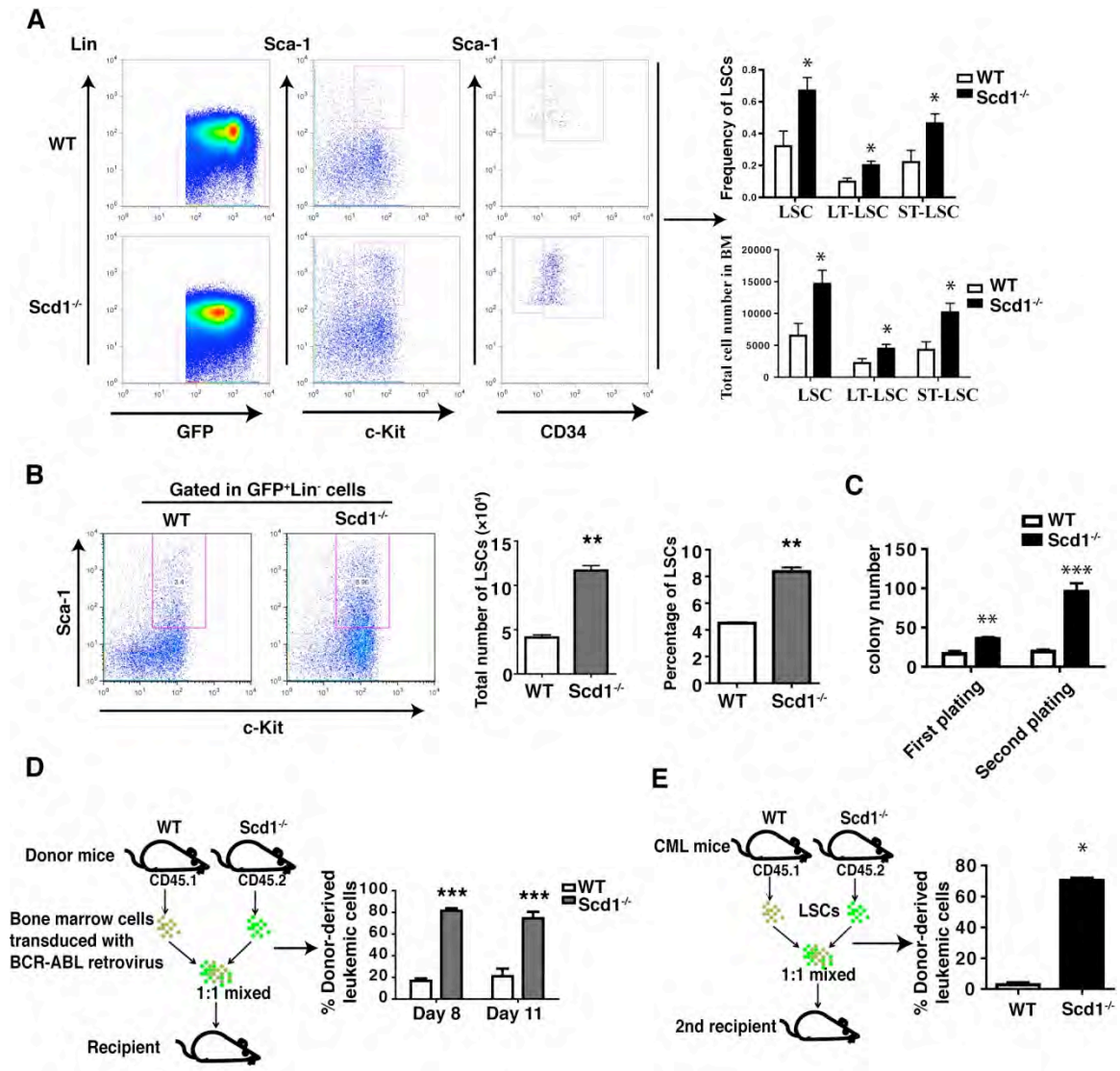
The tumor suppressive effect of Scd1 on CML development could be caused by affecting LSCs. To test this possibility, we first examined whether deletion of Scd1 causes an increase of LSCs in bone marrow of CML mice. Two weeks after CML induction using *BCR-ABL* transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells, we analyzed bone marrow cells from CML mice by FACS (**Fig. 3A**). We found that the percentages and numbers of total LSCs and long-term (LT) (GFP<sup>+</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> CD34<sup>-</sup>) or short-term (ST) (GFP<sup>+</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> CD34<sup>+</sup>) LSCs were significantly higher in recipients of *BCR-ABL*-transduced *Scd1*<sup>-/-</sup> bone marrow cells than in recipients of *BCR-ABL*-transduced WT bone marrow cells (**Fig. 3A**). We also cultured bone marrow cells from CML mice *in vitro* for 7 days under the stem cell culture conditions<sup>232</sup>, and found that the percentage and number of *Scd1*<sup>-/-</sup> LSCs were significantly higher than those of WT LSCs (**Fig. 3B**).

To investigate whether Scd1 affects self-renewal of LSCs, we carried out an *in vitro* serial replating assay to examine the ability of LSCs to form progenitor colonies. We sorted LSCs from recipients of *BCR-ABL*-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells, and plated the cells *in vitro* for colony formation. We observed that *Scd1*<sup>-/-</sup> LSCs formed significantly higher numbers of colonies compared to WT LSCs (**Fig. 3C**). To determine whether deletion of Scd1 causes an increase in LSC function *in vivo*, we mixed *BCR-ABL*-transduced WT (CD45.1) and *Scd1*<sup>-/-</sup> (CD45.2) bone marrow cells in a 1:1 ratio, and transplanted into lethally irradiated mice. At days 8 and 11 after CML induction, FACS analysis showed much higher percentages of CD45.2<sup>+</sup> leukemia cells in

peripheral blood, as compared to the percentages of CD45.1<sup>+</sup> leukemia cells (**Fig. 3D**).

To further confirm this result, LSCs were sorted by FACS from bone marrow of primary CML mice receiving *BCR-ABL*-transduced WT (CD45.1) or *Scd1*<sup>-/-</sup> (CD45.2) bone marrow cells, mixed in a 1:1 ratio, and then transplanted into secondary recipient mice. Eight weeks later, more than 75% of GFP<sup>+</sup>Gr-1<sup>+</sup> leukemia cells in peripheral blood were CD45.2<sup>+</sup> (**Fig. 3E**), indicating that *Scd1*<sup>-/-</sup> LSCs grew predominantly over WT LSCs to induce CML in recipient mice. Together, these results demonstrate that *Scd1* suppresses the function of LSCs.

**Figure 3. *Scd1* suppresses LSCs.** (A) Bone marrow cells from recipients of BCR-ABL-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells were analyzed by FACS at day 14 after BMT. The percentages and numbers of total LSCs, LT-LSCs, ST-LSCs, in bone marrow of recipients of BCR-ABL-transduced *Scd1*<sup>-/-</sup> donor bone marrow cells were dramatically higher as compared to those in bone marrow of recipients of BCR-ABL-transduced WT donor bone marrow cells. (n=4-7 mice per group). Data are representative of one of four independent experiments, and mean values ( $\pm$  s.e.m) are shown (\*, P<0.05). (B) Loss of *Scd1* promotes survival of LSCs *in vitro*. Bone marrow cells from recipients of BCR-ABL-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells were cultured for 6 days under the *in vitro* stem cell culture conditions. The total number and percentage of LSCs were analyzed by FACS. (C) Loss of *Scd1* increased the colony-forming ability of LSCs. The sorted GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells from CML mice were plated into methycellulose media, and the numbers of colonies were counted. The cells from colonies were serially replated. Data are representative of one of two independent experiments. Mean values ( $\pm$  s.e.m) are shown (\*\*, P<0.01; \*\*\*, P<0.001). (D) Equal number of BCR-ABL-transduced bone marrow cells from WT (CD45.1) or *Scd1*<sup>-/-</sup> (CD45.2) mice were mixed well and transplanted into lethally irradiated WT mice (CD45.2). At day 8 and 11 after BMT, FACS analysis gated in GFP<sup>+</sup> cell population showed that the percentages of leukemia cells from *Scd1*<sup>-/-</sup> mice (CD45.2) were dramatically higher than those from WT mice (CD45.1). Mean values ( $\pm$  s.e.m) are shown (\*\*P<0.01; \*\*8P<0.001). (E) Competitive repopulation assay showed that loss of *Scd1* increased engraftment of LSCs. 10<sup>3</sup> sorted LSCs from bone marrow of primary CML mice induced by transplanting BCR-ABL-transduced *Scd1*<sup>-/-</sup> bone marrow cells (CD45.2) and BCR-ABL-transduced WT bone marrow cells (CD45.1) were mixed at 1:1 ratio, followed by transplantation into lethally irradiated recipients mice. The percentages of CD45.2 cells in GFP<sup>+</sup> cell population from PB were compared with those of CD45.2 cells at 8 weeks after BMT (n=3; \*\*\*, P<0.0001).



**Figure 3. *Scd1* suppresses LSCs.**

### Scd1 deletion inhibits apoptosis but not cell cycle of LSCs

To explore cellular mechanisms by which Scd1 regulates LSC function, we investigated whether Scd1 suppresses LSCs through affecting cell cycle progression or apoptosis. We analyzed LSCs from the bone marrow and spleen of CML mice, and we did not observe a significant difference in the percentages of LSCs in the S-G2-M phase of the cell cycle in the presence and absence of *Scd1* (**Fig. 4A**). However, the percentages of apoptotic LSCs (Annexin V<sup>+</sup>) were significantly lower in the absence than in the presence of *Scd1* (**Fig. 4B**), indicating Scd1 suppresses LSCs through reducing their survival. To study the molecular mechanisms by which Scd1 suppresses LSCs and attenuates CML development, we compared expression of *Pten*, *p53* and *Bcl-2* in WT and *Scd1*<sup>-/-</sup> leukemia progenitor cells and LSCs. We found that Scd1 deficiency caused a decreased expression of *Pten* and *p53* and an increased expression of *Bcl-2* in LSCs (**Fig. 4C**) and in GFP<sup>+</sup>Lin<sup>-</sup> leukemia progenitor cells (**Fig. 4D**). Consistently, when we overexpressed Scd1 in LSCs, we found that Scd1 overexpression induced expression of *Pten* and *p53* and reduced expression of *Bcl-2* in LSCs (**Fig. 4E**). To examine functional significance of these genes in LSCs, we constructed the *BCR-ABL-Pten-GFP* retroviral construct to allow co-expression of BCR-ABL, Pten and GFP in the same cells<sup>187</sup>. We transduced bone marrow cells from WT or *Scd1*<sup>-/-</sup> mice with *BCR-ABL-Pten-GFP* retrovirus, and transplanted into lethally irradiated recipient mice. Recipients of *BCR-ABL* transduced *Scd1*<sup>-/-</sup> bone marrow cells displayed higher percentage and number of leukemia cells in peripheral blood compared to recipients of *BCR-ABL* transduced WT bone marrow cells, whereas recipients of *BCR-ABL-Pten* transduced WT and *Scd1*<sup>-/-</sup> bone

marrow cells showed a lower number of leukemia cells (**Fig. 4F, G**). These results indicate that Pten functions downstream of Scd1 to suppress CML development. To further confirm the effect of Pten on *Scd1*<sup>-/-</sup> LSCs, we conducted a colony-forming assay, and found that Pten suppressed colony formation of *Scd1*<sup>-/-</sup> LSCs (**Fig. 4H**).

Using the colony-forming assay, we also examined the function of Bcl2 and p53 through lentiviral shRNA mediated knockdown of Bcl2 and p53 in *Scd1*<sup>-/-</sup> LSCs and Scd1-expressing LSCs, respectively. Western blotting analysis showed that lentiviral shRNA markedly reduced the expression level of Bcl2 and p53 (**Fig. 4I**). Because Scd1 deficiency caused a higher level of Bcl2 expression in *Scd1*<sup>-/-</sup> LSCs (**Fig. 4C**), we assumed that Bcl2 knockdown would reverse the phenotype of *Scd1*<sup>-/-</sup> LSCs. As expected, we found that *Scd1*<sup>-/-</sup> LSCs with Bcl2 knockdown gave rise to less colonies compared to control *Scd1*<sup>-/-</sup> LSCs without Bcl2 knockdown (**Fig. 4J**), indicating that Bcl2 also mediates the function of Scd1 in LSCs. Furthermore, we knocked down p53 in Scd1-expressing LSCs and observed increased number of colonies (**Fig. 4K**), although the increase was not statistically significant, suggesting that p53 plays a minor role downstream of Scd1 in LSCs.

**Figure 4. *Scd1* deletion promotes survival but not cell cycle progression of LSCs. (A)**

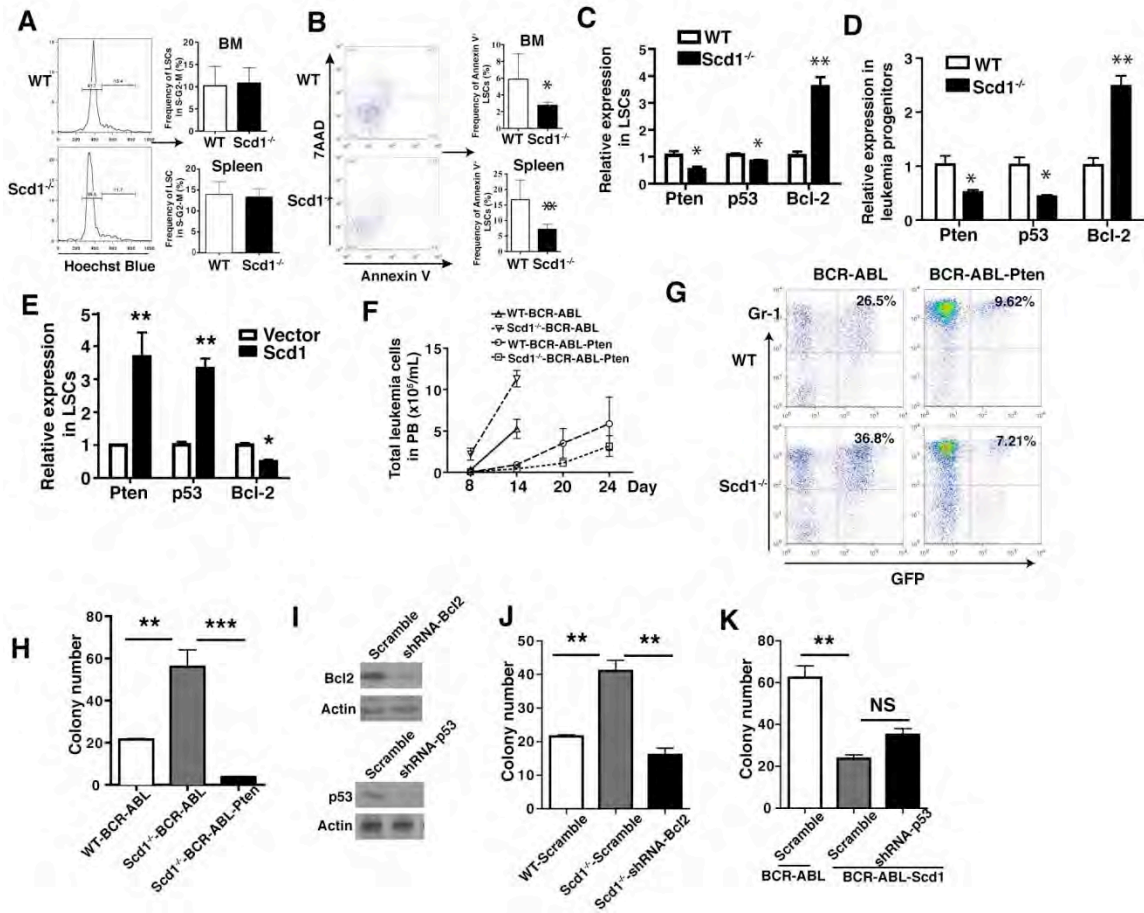
The cell cycle analysis showed comparable percentage of cycling LSCs from the bone marrow and spleen of CML mice receiving *BCR-ABL*-transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells. Data shown are a representative experiment from three independent experiments, with 4-7 mice per group in each experiment. Mean values ( $\pm$  s.e.m) are shown (NS, no significance). **(B)** Apoptotic analysis of LSCs from the bone marrow and spleen of CML mice receiving *BCR-ABL*-transduced wild type or *Scd1*<sup>-/-</sup> bone marrow cells. Data shown are a representative experiment from three independent experiments, with 4-7 mice per group in each experiment. Mean values ( $\pm$  s.e.m) are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). **(C and D)** Real time RT-PCR analysis of expression of *Pten*, *p53*, and *Bcl-2* in the sorted WT or *Scd1*<sup>-/-</sup> LSCs and progenitor cells from CML mice. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). **(E)** Real time RT-PCR analysis showed that *Scd1* up-regulated expression of *Pten* and *p53* and down-regulated expression of *Bcl-2* in LSCs. Bone marrow cells from 5-FU pretreated mice were co-transduced with *MSCV-BCR-ABL-GFP* and *MSCV-Scd1-hCD4* or *MSCV-hCD4*. 14 days after BMT, *Scd1*-overexpressing LSCs ( $hCD4^{+}GFP^{+}Lin^{-}Sca-1^{+}c-Kit^{+}$ ) or control LSCs were sorted for extracting total RNA for real time PCR analysis. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). **(F)** FACS analysis showing the total numbers of leukemia cells in PB of recipients of *BCR-ABL* or *BCR-ABL-Pten* transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells at Day 14 after BMT. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). **(G)** FACS analysis showing the percentages of leukemia cells in PB of recipients of *BCR-ABL* or *BCR-ABL-Pten* transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells at Day 14 after BMT. **(H)** Colony-forming assay showed that *Pten* overexpression reduced colony-forming ability of *Scd1*<sup>-/-</sup> LSCs. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). **(I)** Western blotting analysis of *Bcl2* and *p53* expression after lentivirus shRNA mediated knockdown. **(J)** Colony-forming assay. CML bone marrow cells from recipients of *BCR-ABL* transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells were infected with shRNA-*Bcl2* or control lentivirus and cultured in the presence of puromycin for 48 hours. Equal numbers of infected cells were plated into methycellulose media. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*\*,  $P < 0.01$ ). **(K)** Colony-forming assay. CML bone marrow cells from recipients of *BCR-ABL* or *BCR-ABL-Scd1* transduced WT bone marrow cells were infected with shRNA-*p53* or control lentivirus and cultured in the presence of puromycin for 48 hours. Equal numbers of infected cells were plated into methycellulose media. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*\*,  $P < 0.01$ ; NS, no significance).



### **Scd1 regulates *Pten*, *p53* and *Bcl2* transcription and mRNA stability**

To examine whether Scd1 regulates expression of *Pten*, *p53*, and *Bcl2* at a transcriptional or post-transcriptional level, we first performed chromatin immunoprecipitation of RNA Pol-II using WT and *Scd1*<sup>-/-</sup> leukemia cells and found that the enrichments of Pol-II in the promoters of *Pten* and *p53* were significant higher in the WT leukemia cells than those in the *Scd1*<sup>-/-</sup> leukemia cells (**Fig. 5A, B**). We also found that the enrichment of Pol-II in the promoter of *Bcl2* was lower in the WT leukemia cells than that in the *Scd1*<sup>-/-</sup> leukemia cells (**Fig. 5C**). These data suggest that Scd1 deletion reduces the transcription of *Pten* and *p53* and induces *Bcl2* transcription. We tested whether Scd1 affects mRNA stability of *Pten*, *p53* and *Bcl2*. Leukemia cells from WT and *Scd1*<sup>-/-</sup> CML mice were treated with actinomycin D (5µg/mL) to block the transcription, and total RNA was subsequently harvested at various time points and the mRNA half-life for *Pten*, *p53*, or *Bcl2* was examined by RT-PCR. We found that the mRNA half-life for *Pten* and *p53* was similar in WT and *Scd1*<sup>-/-</sup> leukemia cells, but the mRNA half-life for *Bcl2* was markedly increased in *Scd1*<sup>-/-</sup> leukemia cells (**Fig. 5D**). These results indicate that Scd1 regulates expression of *Pten* and *p53* only at the transcriptional level, but regulates expression of *Bcl2* through affecting both transcription and mRNA stability.

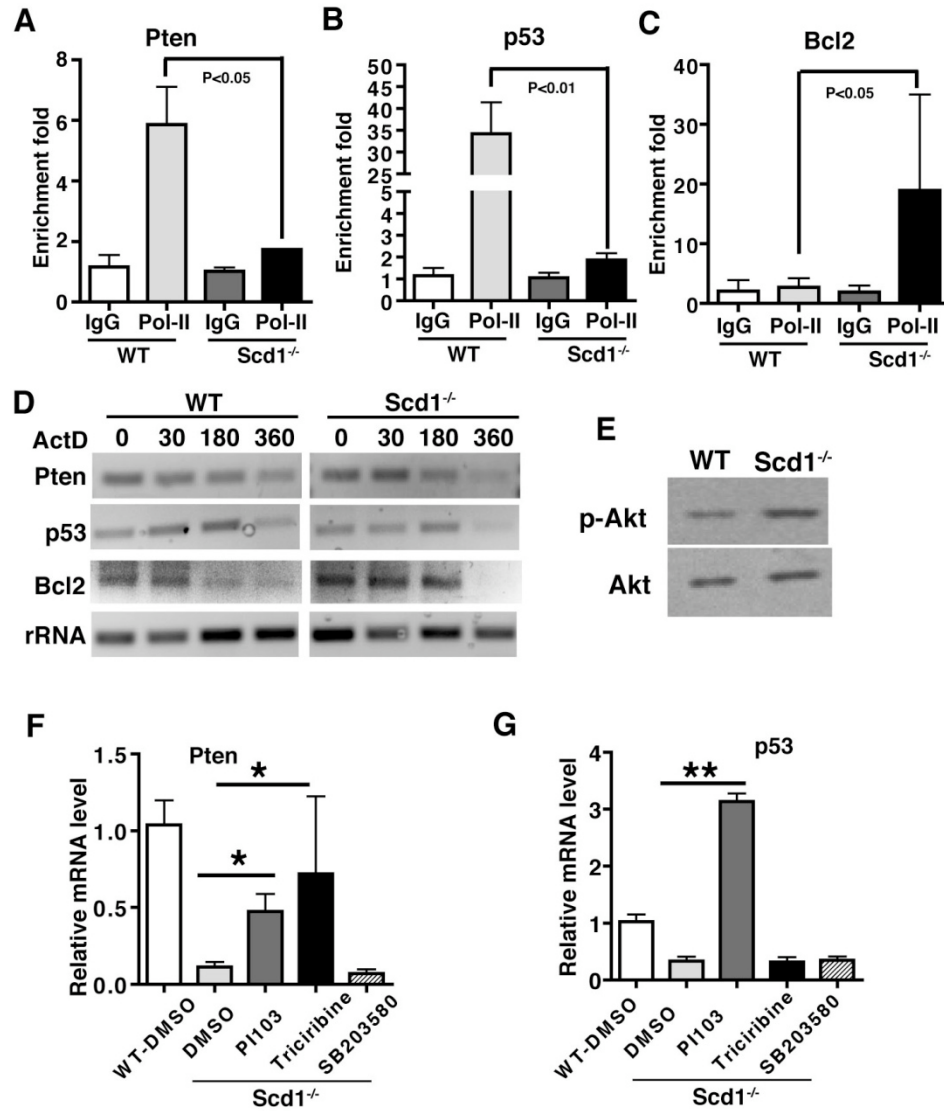
To further study the functional relationship between Scd1 and *Pten/p53*, we examined Akt phosphorylation in *Scd1*<sup>-/-</sup> leukemia cells, because a published study showed an increased Akt activity in *Scd1*<sup>-/-</sup> cells<sup>173</sup>. We found that the level of Akt phosphorylation level was elevated in *Scd1*<sup>-/-</sup> leukemia cells (**Fig. 5E**). Therefore, we



**Figure 4.** Scd1 deletion promotes survival but not cell cycle progression of LSCs.

asked further whether Akt pathway is involved in the regulation of Pten and p53 by Scd1. We treated *Scd1*<sup>-/-</sup> CML leukemia cells with Akt inhibitor triciribine, and found that inhibition of Akt activity partially reversed expression of *Pten* but not *p53* (**Fig. 5F, G**). Inhibition of PI3K/mTOR pathway by the dual inhibitor PI103 reversed expression of both *Pten* and *p53* (**Fig. 5F, G**). However, inhibition of MAPK activity by the inhibitor SB203580 did not have an effect on expression of either *Pten* or *p53* (**Fig. 5F, G**). These results suggest that the PI3K/Akt but not MAPK pathway mediates the regulation of Pten and p53 by Scd1 .

**Figure 5. Scd1 regulates *Pten*, *p53* and *Bcl2* transcription and mRNA stability.** (A and B) Pol-II ChIP analysis showed the accumulation of polymerase II in the promoters of *Pten* and *p53* of WT but not *Scd1*<sup>-/-</sup> leukemia cells. (C) Pol-II ChIP analysis showed the accumulation of polymerase II in the promoter of *Bcl2* of *Scd1*<sup>-/-</sup> but not WT leukemia cells. (D) Decay time course of the mRNA levels for *Pten*, *p53* and *Bcl2* in WT and *Scd1*<sup>-/-</sup> leukemia cells. (E) Western blot analysis showed Akt phosphorylation in WT and *Scd1*<sup>-/-</sup> leukemia progenitor cells. (F) Real time RT-PCR assay for *Pten* expression in *Scd1*<sup>-/-</sup> leukemia cells treated with PI103, triciribine and SB203580. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (G) Real time RT-PCR assay for *p53* expression in *Scd1*<sup>-/-</sup> leukemia cells treated with PI103, triciribine and SB203580. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ )



**Figure 5. Scd1 regulates *Pten*, *p53* and *Bcl2* transcription and mRNA stability**

### Induction of Scd1 expression inhibits LSCs and attenuates CML development

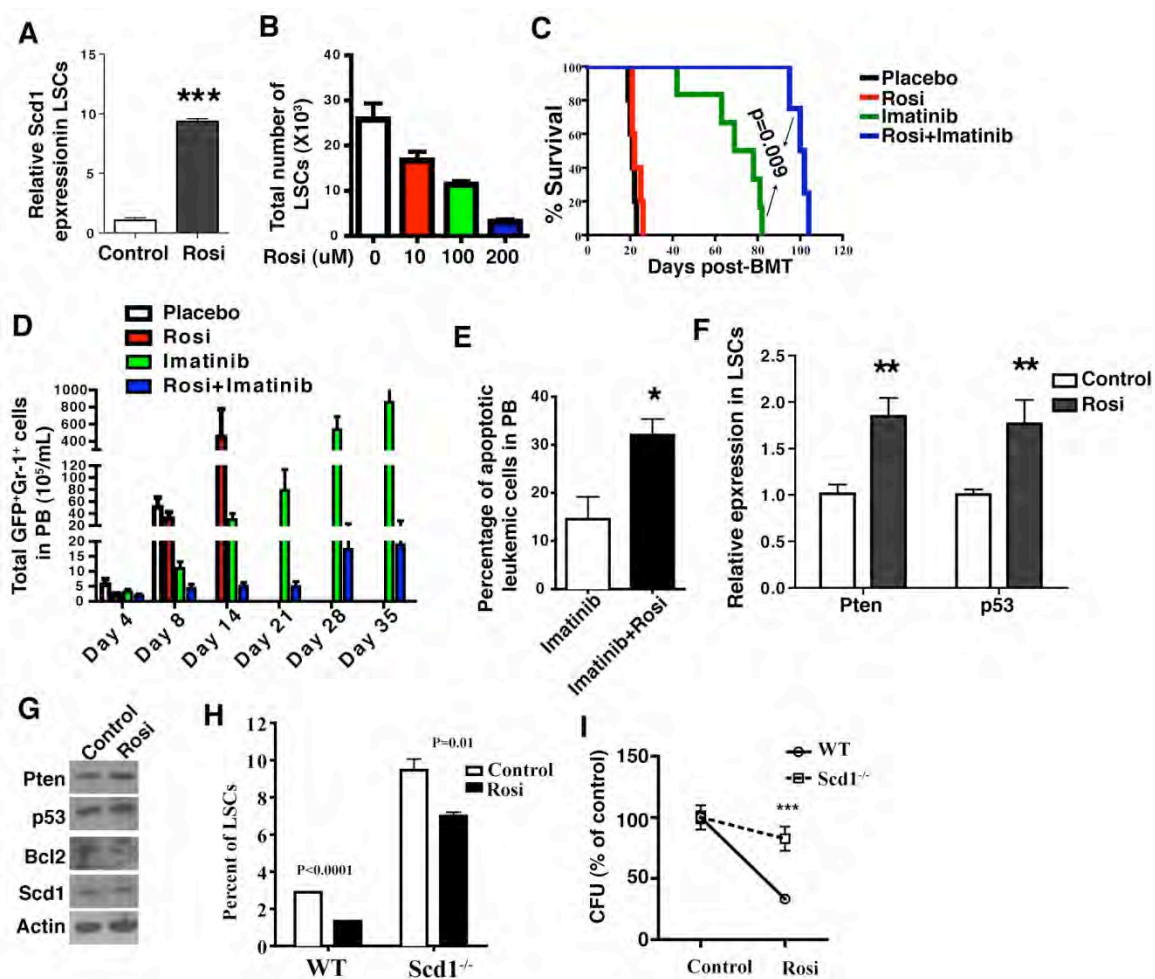
The tumor suppressive role of Scd1 in CML development prompted us to test whether upregulation of Scd1 expression delays CML development. It has been shown that the PPAR $\gamma$  agonists, rosiglitazone and pioglitazone, induce Scd1 expression, and this Scd1 upregulation is blocked by PPAR $\gamma$  antagonists *in vitro* and *in vivo*<sup>236,237</sup>. Therefore, we treated bone marrow cells from CML mice with rosiglitazone for 6 days *in vitro*, and found that *Scd1* expression was significantly induced in LSCs (**Fig. 6A**), accompanied by a dramatic decrease in the numbers of LSCs (**Fig. 6B**). To test the effect of rosiglitazone on CML development, we treated CML with a placebo, imatinib alone, rosiglitazone alone, and the two agents in combination. Although treatment with rosiglitazone alone did not prolong the survival of CML mice, combined treatment with rosiglitazone and imatinib synergized and more significantly prolonged survival of CML mice than did imatinib alone (**Fig. 6C**). This therapeutic effect by rosiglitazone and imatinib correlated with much lower numbers of GFP<sup>+</sup>Gr-1<sup>+</sup> myeloid leukemia cells (**Fig. 6D**) and increased apoptosis (**Fig. 6E**) of leukemia cells in peripheral blood. Because Scd1 overexpression induced expression of *Pten* and *p53* in LSCs (**Fig. 4E**), we tested whether rosiglitazone treatment affects *Pten* and *p53* expression. We observed higher levels of *Pten* and *p53* expression in LSCs after rosiglitazone treatment (**Fig. 6F**). We also treated human CML cell line K562 with rosiglitazone for 24 hours, and found that rosiglitazone treatment induced expression of *Pten*, *p53* and *Scd1* but decreased expression of *Bcl2* (**Fig. 6G**).

To confirm the requirement of Scd1 for the effect of rosiglitazone in suppressing leukemia development, we cultured bone marrow cells from recipients of *BCR-ABL*

transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells *in vitro* under the stem cell culture conditions in the presence of rosiglitazone. We found that rosiglitazone treatment caused a marked decrease (up to 55%) of WT LSCs (**Fig. 6H**). In contrast, rosiglitazone treatment only caused a modest decrease in the percentage of *Scd1*<sup>-/-</sup> LSCs (25% decrease) (**Fig. 6H**), indicating that rosiglitazone regulates LSCs through Scd1. Additionally, we compared the impact of rosiglitazone on the colony-forming ability between WT and *Scd1*<sup>-/-</sup> LSCs. Since *Scd1*<sup>-/-</sup> LSCs had an increased colony-forming ability (**Fig. 3C**), we normalized the colony number in the control group (DMSO) to 100%, and showed that the deletion of Scd1 caused a slight reduction in colony formation after rosiglitazone treatment (**Fig. 6I**), further demonstrating that the inhibitory effect of rosiglitazone on LSCs is mediated through induction of Scd1 expression..

**Figure 6. Induction of *Scd1* expression suppresses LSCs and attenuates CML development.** (A) Real time RT-PCR showed the induction of *Scd1* expression by rosiglitazone in LSCs. Bone marrow cells from primary CML mice were treated with rosiglitazone *in vitro* for 7 days, and LSCs were sorted by FACS and total RNA was isolated for RT-PCR analysis. Representative data from two independent experiments are shown. Mean values ( $\pm$  s.e.m) are shown (\*\*\*,  $P < 0.001$ ). (B) Bone marrow cells from primary CML mice were treated with rosiglitazone *in vitro* for 7 days, and the number of LSCs was decreased upon rosiglitazone treatment. (C) Kaplan-Meier survival curve for CML mice treated with a placebo, rosiglitazone alone, imatinib alone, or both rosiglitazone and imatinib. (n=6-12 mice per group;  $P = 0.009$ ). (D) The total numbers of leukemia cells in PB of CML mice treated with a placebo, rosiglitazone alone, imatinib alone, or both rosiglitazone and imatinib were analyzed at day 4, 8, 14, 21, 28 and 35 after initiation of the treatments. Results are representatives of two independent experiments. (E) FACS analysis of the percentages of apoptotic leukemia cells in PB of CML mice treated with imatinib alone (n=5), or both rosiglitazone and imatinib (n=5) for 8 weeks. Mean values ( $\pm$  s.e.m) are shown (\*,  $P < 0.05$ ). (F) Rosiglitazone treatment induced expression of *Pten* and *p53* in LSCs *in vitro*. Mean values ( $\pm$  s.e.m) are shown (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). (G) Western blot analysis showed the regulation of expression of *Pten*, *p53*, *Bcl2* and *Scd1* by rosiglitazone in human K562 cells. (H) The percentages of WT and *Scd1*<sup>-/-</sup> LSCs cultured *in vitro* in the presence of rosiglitazone. (I) *Scd1* deletion reduced the inhibitory effect of rosiglitazone on colony formation of LSCs. Bone marrow cells from recipients of *BCR-ABL* transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells were plated in methycellulose media in the presence of rosiglitazone. Note that the colony forming unit for the cells treated with DMSO was defined as 100%.



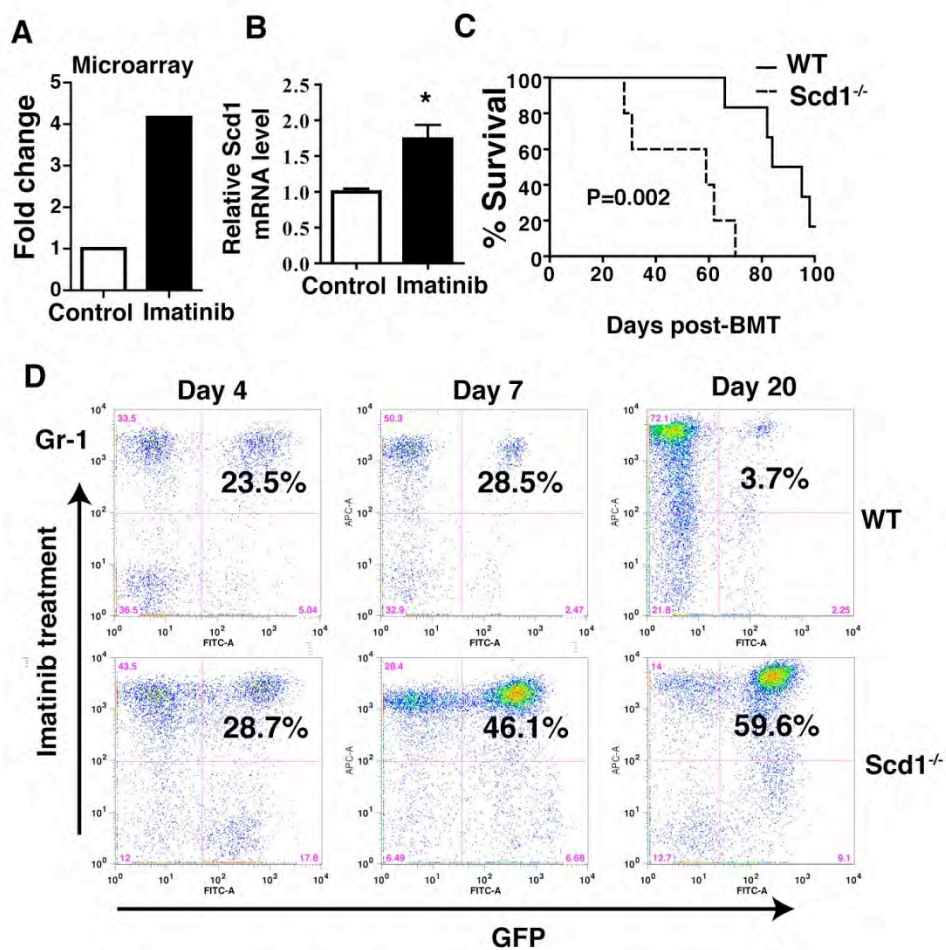


**Figure 6. Induction of Scd1 expression suppresses LSCs and attenuates CML development**

### **Scd1 mediates the inhibitory effect of imatinib on CML development**

To investigate whether Scd1 synergizes with imatinib in delaying CML development, we first analyzed our LSC microarray data and found that imatinib treatment induced *Scd1* expression in LSCs (**Fig. 7A**). We next treated leukemia cells with imatinib *in vitro* under the stem cell culture conditions, and found that treatment with imatinib or rosiglitazone alone caused an increase in *Scd1* expression, and treatment with both drugs further induced Scd1 expression (**Fig. 7B**). Furthermore,, we treated recipients of *BCR-ABL* transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells with imatinib, and found that upon imatinib treatment, survival of recipients of *BCR-ABL* transduced WT bone marrow cells was significantly longer than recipients of *BCR-ABL* transduced *Scd1*<sup>-/-</sup> bone marrow cells (**Fig. 7C**), consistent with a lower percentage of leukemia cells in the presence of Scd1 (**Fig. 7D**).

**Figure 7. *Scd1* mediates the inhibitory effect of imatinib on CML development.** (A) Microarray analysis showed that imatinib treatment induced *Scd1* expression in LSCs. (B) Real time RT-PCR showed that imatinib treatment induced *Scd1* expression in leukemia cells. (\*,  $P < 0.05$ ). (C) Kaplan-Meier survival curve for imatinib treated recipients of BCR-ABL transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells (n=5-8 mice per group;  $P = 0.002$ ). (D) FACS analysis showed the percentages of leukemia cells in PB of recipients of BCR-ABL transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells at day 4, 7 and 20 after imatinib treatment.



**Figure 7. Scd1 mediates the inhibitory effect of imatinib on CML development**

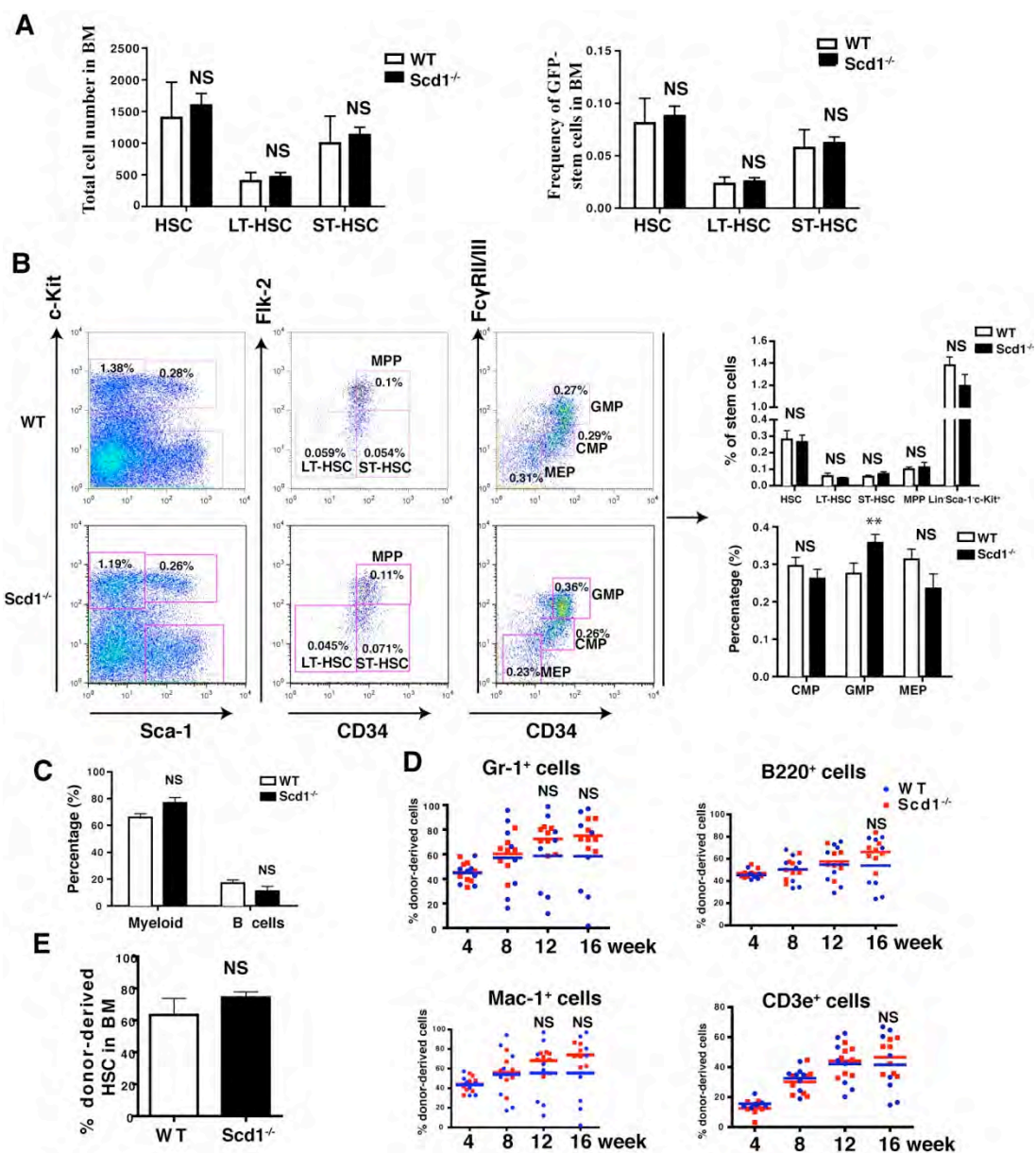
### Scd1 does not affect normal HSCs

Scd1 is a suppressor of LSCs in CML mice (Figure 3), we wondered whether this inhibitory effect is specific to LSCs with no effect on normal HSCs. We analyzed non-BCR-ABL-expressing (GFP<sup>-</sup>) HSCs (GFP<sup>-</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>), LT- (GFP<sup>-</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>-</sup>) and ST- (GFP<sup>-</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>) HSCs in CML mice receiving *BCR-ABL*-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells. We did not observe any significant differences in the percentages and numbers of these cell populations between the two groups (**Fig. 8A**). This result suggests that Scd1 does not suppress normal HSCs. To confirm this observation, we directly analyzed HSCs and progenitors in bone marrow of WT and *Scd1*<sup>-/-</sup> mice. We found that the percentages of total HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>), LT-HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>-</sup>Flk-2<sup>-</sup>), ST-HSCs (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>Flk-2<sup>-</sup>), and MPP (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>Flk-2<sup>+</sup>) in bone marrow of *Scd1*<sup>-/-</sup> mice were similar to those in WT mice (**Fig. 8B**). Although the percentage of GMP (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>FcyRII/III<sup>hi</sup>) was modestly higher in *Scd1*<sup>-/-</sup> mice than that in WT mice, the percentages of CMP (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>FcyRII/III<sup>lo</sup>) and MEP (Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>-</sup>FcyRII/III<sup>lo</sup>) in bone marrow were similar between *Scd1*<sup>-/-</sup> and WT mice (**Fig. 8B**). In addition, *Scd1*<sup>-/-</sup> mice had a normal distribution of mature myeloid cells (Gr-1<sup>+</sup>Mac-1<sup>+</sup>) and B lymphoid cells (B220<sup>+</sup>) (**Fig. 8C**).

To determine whether *Scd1* affects the self-renewal capacity of HSCs, we did a competitive repopulation assay. 2 x 10<sup>5</sup> bone marrow cells from WT (CD45.2) or *Scd1*<sup>-/-</sup> (CD45.2) mice were transplanted into lethally irradiated CD45.1 mice along with equal number of WT CD45.1 competitor bone marrow cells. Analysis of these chimeric mice

was done by FACS during 1-4 months after the bone marrow transplantation. We found that the percentages of WT and *Scd1*<sup>-/-</sup> donor-derived myeloid cells (Gr-1<sup>+</sup>, Mac-1<sup>+</sup>), B cells (B220<sup>+</sup>), and T cells (CD3e<sup>+</sup>) in peripheral blood were similar at 4 and 8 weeks after BMT; however the percentage of *Scd1*<sup>-/-</sup> donor-derived myeloid cells at 12 and 16 weeks, and the percentages of *Scd1*<sup>-/-</sup> donor-derived B and T cells at 16 weeks were slightly higher than those of WT donor-derived cells (**Fig. 8D**), although the difference was not statistically significant. At 16 weeks, we directly compared the percentages of donor-derived WT and *Scd1*<sup>-/-</sup> HSCs in bone marrow, and we did not observe a significant difference (**Fig. 8E**). Together, these results indicate that *Scd1* does not affect the function of normal HSCs.

**Figure 8. *Scd1* deficiency does not affect the function of normal HSCs.** (A) The total numbers and percentages of HSCs (GFP<sup>-</sup> Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>), LT-HSCs (GFP<sup>-</sup> Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>-</sup>), and ST-HSCs (GFP<sup>-</sup> Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>) in the GFP<sup>-</sup> cell population of recipients of *BCR-ABL* transduced WT and *Scd1*<sup>-/-</sup> bone marrow cells were compared. (NS, non significance) (B) The percentages of normal stem cells and progenitors from WT and *Scd1*<sup>-/-</sup> bone marrow (n=4) were compared. HSC: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>; LT-HSC: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>-</sup>Flk-2<sup>-</sup>; ST-HSC: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>Flk-2<sup>-</sup>; MPP: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>Flk-2<sup>+</sup>; Progenitor: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>-</sup>; CMP: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>-</sup>CD34<sup>+</sup>FcγRII/III<sup>lo</sup>; GMP: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>-</sup>CD34<sup>+</sup>FcγRII/III<sup>hi</sup>; MEP: Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>-</sup>CD34<sup>-</sup>FcγRII/III<sup>lo</sup>. (C) The percentages of myeloid (Gr-1<sup>+</sup>) and B cells (B220<sup>+</sup>) in bone marrow of WT and *Scd1*<sup>-/-</sup> mice were compared. Mean values (± s.e.m) are shown. (D) Competitive repopulation assay for the effect of *Scd1* on normal HSCs. FACS analysis of the percentage of donor-derived (CD45.2) myeloid (Gr-1<sup>+</sup>, Mac-1<sup>+</sup>), B cells (B220<sup>+</sup>), T cells (CD3e<sup>+</sup>) in PB of recipients of normal bone marrow cells from WT and *Scd1*<sup>-/-</sup> mice. (E) FACS analysis of the donor-derived (CD45.2<sup>+</sup>) HSCs in the bone marrow of recipients of WT and *Scd1*<sup>-/-</sup> mice. (n=8 mice per group) Mean values (± s.e.m) are shown.



**Figure 8. Scd1 deficiency does not affect the function of normal HSCs**



## Discussion

Our study supports a critical role for *Scd1* in CML development through specifically regulating LSCs but not normal HSCs. In our previously published study, we identified another lipid-metabolic gene, arachidonate 5-lipoxygenase (*Alox5*), required for functional regulation of LSCs but not normal HSCs<sup>85</sup>. Our identification of *Scd1* in this study strengthens the feasibility to specifically target LSCs in the treatment of CML.

Although dysregulated expression of *Scd1* has been observed in several human cancers<sup>238</sup>, to our knowledge this study provides the first evidence for the role of *Scd1* in hematopoiesis and leukemogenesis. Previous data indicated *Scd1* regulated cancer cell proliferation and survival. Knockdown *Scd1* by siRNA significantly reduced the survival of multiple human tumor cell lines<sup>239</sup>. In contrast, in this study, we find that *Scd1* plays a tumor suppressive role in LSCs through regulating apoptosis of LSCs. This indicates that *Scd1* plays different roles in different cancers. Additionally, while CML development was accelerated in the loss of *Scd1*, the development of Ph<sup>+</sup> B-ALL could be unimpaired. It is known that CML is a stem cell disease while the cell of origin for ALL is a committed B cell progenitor<sup>23</sup>. Therefore, the distinct roles of *Scd1* in different leukemia subtypes suggest that *Scd1* acts in a cell type-specific manner.

With respect to the molecular basis for the functional regulation of LSCs by *Scd1*, we show that *Scd1* deficiency results in dysregulated expression of apoptosis-related genes, including decreased expression of *Pten* and *p53*, and increased expression of *Bcl-2*, which is consistent with the observation from other groups showing that *Scd1* deficiency reduces cardiac apoptosis in ob/ob mice due to increased expression of

antiapoptotic factor *Bcl-2*<sup>240</sup>. In addition, we also show that treatment with PPAR $\gamma$  agonist induces the apoptosis of leukemia cells, which is associated with a higher expression of *Scd1*, *Pten* and *p53*. A recent study showed that unsaturated fatty acid affected *Pten* expression in hepatoma cells through regulating microRNA mediated *Pten* mRNA stability, which depends on the mTOR and NF- $\kappa$ B pathways<sup>241</sup>. Our results show that *Scd1* regulates *Pten*, *p53* and *Bcl2* at the transcriptional and/or post-transcriptional level. However, detailed mechanisms by which *Scd1* regulates expression of *Pten*, *p53* and *Bcl2* need to be further investigated in the future.

### Material and methods

**Mice.** *Scd1*<sup>-/-</sup>, C57BL/6J-CD45.1, and C57BL/6J-CD45.2 mice were obtained from mice were purchased from The Jackson Laboratory. All mice were in C57BL/6J background, and were bred and maintained in a temperature- and humidity-controlled environment and given unrestricted access to 6% chow diet and acidified water.

**Generation of retrovirus stocks.** The retroviral constructs *MSCV-IRES-GFP*, *MSCV-BCR-ABL-IRES-GFP*, *MSCV-BCR-ABL-IRES-Scd1*, *MSCV-Scd1-IRES-humanCD4* *MSCV-BCR-ABL-IRES-Pten-IRES-GFP* were used to generate high-titer, helper-free, replication-defective ecotropic virus stock by transient transfection of 293T cells as previously described<sup>23</sup>.

### Lentiviral transduction and shRNA-mediated knockdown of p53 and Bcl2.

Lentiviral shRNA vector pLKO.1 were purchased from OpenBiosystems. Sequences of *p53* and *Bcl2* shRNA were as follows: shRNA-*p53*: sense 5'- ccagtctacttcccgccataa-3':

shRNA-p53 antisense 5'-ttatggcgggaagtagactgg-3'; shRNA-Bcl2 sense 5'-cgtgatgaagtacatacatta -3'; shRNA-Bcl2 antisense 5'-taatgtatgtacttcacac-3'. For transduction of shRNAs, bone marrow cells from CML mice were transfected with lentiviral shRNA, and 24 hours later, were selected under puromycin (2.5µg/ml) for 48 hours to enrich shRNA-expressing cells.

**Bone marrow transduction/transplantation.** 8 to 12 week-old C57BL/6 mice were used for bone marrow transduction/transplantation. Retroviral transduction and transplantation of mouse bone marrow cells for inducing CML and B-ALL by BCR-ABL had been described previously<sup>23,86,232</sup>. Diseased mice were evaluated by flow cytometric, histopathological and molecular analyses. For secondary transplantation, equal number of bone marrow cells from primary CML mice was transplanted into lethally irradiated recipient mice. For drug treatment, rosiglitazone (GlaxoSmithKline) and imatinib (Novartis) were given orally in a volume of less than 0.3 mL by gavage (100mg/kg, twice a day for rosiglitazone, and 100mg/kg, twice a day for imatinib) beginning at 8 days after BMT, and continuing until the morbidity or death of the mice with leukemia.

**Flow cytometry analysis.** Hematopoietic cells were collected from bone marrow and peripheral blood of CML mice for FACS analysis. For stem cell analysis, five mice from each experimental group were sacrificed at day 14 after BMT to collect bone marrow cells. Red blood cells (RBCs) were depleted using RBC lysis buffer (containing NH<sub>4</sub>Cl, KHCO<sub>3</sub>, and EDTA). To stain the cells with antibodies, bone marrow cells were suspended in staining medium (Hank's Balanced Salt Solution (HBSS) with 2% heat-inactivated calf serum), and incubated with biotin-labeled lineage antibody cocktail

containing a mixture of antibodies against CD3, CD4, CD8, B220, Gr-1, Mac-1 and Ter119 at 4°C for 15 min. After washing, the fluorochrome-labeled secondary antibody (APC-Cy7-conjugated Streptavidin) for recognizing biotin and PE-conjugated c-Kit and APC-conjugated Sca-1 antibodies were added to the cells for 15min at 4°C in the dark. Long-term and short-term LSCs were distinguished by the CD34 antibody. CML stem cell population (GFP<sup>+</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>) was analyzed by FACS. All these antibodies were purchased from eBioscience.

Cell cycle analysis of LSCs was performed by staining cells with antibodies in combination with Hoechst 33342 for 90 min at 37°C, followed by flow cytometry analysis. To analyze apoptosis of bone marrow cells, the cells were stained with Annexin V (eBioscience) for 15 min at room temperature. 7AAD was added before flow cytometry analysis.

**Leukemia stem cell culture.** For mouse leukemia stem cell culture, bone marrow cells isolated from CML mice were cultured in vitro in the presence of stemspan SFEM, SCF, IGF-2, TPO, heparin, and  $\alpha$ -FGF as reported previously for culture of hematopoietic stem cells. For in vitro treatment, rosiglitazone (Cayman Chemical) was dissolved in DMSO.

***In vitro* methylcellulose colony formation assay.**

LSCs (GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) from bone marrow of CML mice receiving BCR-ABL-transduced WT or *Scd1*<sup>-/-</sup> bone marrow cells were sorted by FACS, and were cultured in methylcellulose medium (Methocult GF M3434; Stem Cell Technologies) at 37°C in humidified air for 7 days. Colonies were counted under microscope.

**Chromatin immunoprecipitation of RNA Polymerase II.** ChIP assays were performed as previously described (Sheng et al., 2009). Briefly,  $3 \times 10^7$  leukemia cells from WT and *Scd1*<sup>-/-</sup> CML mice were incubated with 1% formaldehyde for 10 min at room temperature before crosslinking was quenched by addition of 0.125 M glycine. Cells were collected by centrifugation and lysed in lysis buffer containing 50mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, proteinase inhibitors and phosphatase inhibitors. The cells suspension was sonicated seven times for 10s each with 2-min intervals on ice using a Misonix Sonicator 3000 at output 8. Sonicated chromatin was then incubated at 4°C overnight with 5 µg polymerases II antibody (Avaon). Immunoprecipitated DNA was amplified by real-time PCR using the following primers: Pten promoter-sense (5'-cgggactctttgtgcactg-3'), Pten promoter-antisense (5'-gcggctcaactctcaaactt-3'), p53 promoter-sense (5'-aacagtggcgggtccacttac-3'), and p53 promoter-antisense (5'-gggacttgccagagtcaggat-3'), Bcl2 promoter-sense (5'-ccggcggagaatgaagtaa-3'), and Bcl2 promoter-antisense (5'-cgcaccctttctcctcct-3').

**Real time RT-PCR.** Total RNA was isolated from GFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> bone marrow cells from mice using the RNeasy Mini kit (Qiagen, CA). cDNA was synthesized using the Ovation-Pico cDNA synthesis method. All real time PCR reactions were done using the Applied Biosystems 7500. 25µL reaction system was composed of 12.5µL SYBR Green, 2.5µL 20uM primer mixture, 10ng cDNA and nuclease-free water. All experiments were performed in triplicate. Beta-actin was used as the internal control. The primer sequences were listed as following: Scd1: sense: 5'-ttcttacacgaccaccacca-3'; antisense: 5'-gcaggagggaaccagtatga-3'; Pten: sense: 5'-acaccgcaaatttaactgc-3';

antisense: 5'-tacaccagtcggtccctttc-3'; p53: sense: 5'-agagaccgccgtacagaaga-3'; antisense: 5'-ctggtagcatgggcatccttt-3'; Bcl2: sense: 5'-ctggcttcttccttccag-3'; antisense: 5'-gacggtagcgacgagaggaag-3';  $\beta$ -actin: sense: 5'-atggaatcctgtggcatcca-3'; antisense: 5'-cgctcaggaggagcaatgat-3'.

**Statistical analysis.** Results are given as mean  $\pm$  s.e.m. Statistical analysis was performed by Students' t test. For survival curves, p values were obtained using a Log-rank test.

## CHAPTER V

### FINAL SUMMARY AND DISCUSSION

Studies on CML from the identification of the first cancer-associated chromosomal abnormality ( $\text{Ph}^+$ ) to the subsequent development of TKIs that inhibit BCR-ABL kinase activity in CML have served as a paradigm for cancer research and therapy. Although TKIs have achieved great success in the treatment of CML patients, of which 87% of chronic phase CML patients treated with imatinib as initial therapy show a complete cytogenetic response after 5 years of follow-up<sup>43</sup>, resistance to TKI and the subsequent disease relapse have been frequently observed in patients upon discontinuation of this therapy, especially in patients with advanced-stage disease<sup>59,61,242</sup>. Two major reasons for the development of resistance are generally agreed. The first reason is the mutations in the BCR-ABL kinase domain that lead to the change of the BCR-ABL kinase domain conformation, thereby blocking the inhibition of BCR-ABL kinase activity by TKIs. Studies on the structure and dynamics of c-ABL/BCR-ABL, along with the development of computational biology, have led to the development of alternative strategies to target other BCR-ABL motifs. In this regard, several inhibitors have been developed. A highly selective non-ATP competitive inhibitor of BCR-ABL, GNF-2/5, which can target the C-terminal myristoyl pocket of ABL, was identified through a high-throughput screen<sup>54,55</sup>. DCC-2036, which regulates the conformational control inhibition and potently inhibits both unphosphorylated and phosphorylated ABL1 through inducing an inactive conformation, exhibits efficacy against the T315I mutant in

vivo<sup>56</sup>. An engineered protein 7c12, which disrupts the SH2-kinase domain interface, inhibits BCR-ABL kinase activity and leukemogenesis<sup>243</sup>. As these motifs are remote from the kinase domain and would be unaffected by kinase mutations that confer the ability of TKI-resistance, BCR-ABL bearing mutations in the kinase domain exhibit higher sensitivity to these inhibitors. This approach helps to overcome drug resistance resulting from BCR-ABL kinase domain mutations.

The second reason for the development of resistance to TKIs and subsequent disease relapse is the existence of a rare population of LSCs resistant to TKIs. It has been shown that LSCs in CML are insensitive to imatinib<sup>62</sup>, and cannot be eliminated completely by TKIs in CML mice and patients<sup>63,64</sup>. Eradication of LSCs appears to be extremely difficult, calling for a greater understanding of the biology of LSCs. Studies from our group and others have revealed that LSCs of CML form a hierarchy similar to that seen in normal hematopoiesis, in which a rare stem cell population with limitless self-renewal potential gives rise to progenies that lack such potential<sup>33,63</sup>. However, we hypothesize that LSCs possess biological features that are different from those of HSCs and are critical for their malignant characteristics. Therefore, based on these concerns, in my thesis work, using BCR-ABL-induced CML as a disease model for LSCs, I attempted to understand the molecular mechanisms of LSC maintenance by comparing the gene expression profiling of normal HSCs and LSCs. I found that the HIF1 $\alpha$  signaling pathway positively regulates the self-renewal of LSCs. I also identified two novel negative regulators, Blk and Scd1, which play tumor suppressor roles in LSCs.

### **The positive regulatory pathway---HIF1 $\alpha$ in LSCs**



In this part of my work, I found that the HIF1 $\alpha$  signaling pathway is active in BCR-ABL-expressing LSCs compared to normal HSCs. Recent studies showed that HIF1 $\alpha$  plays an important role in cancer progression by activating the transcriptional programs necessary for maintaining the ability of self-renewal and multipotency of cancer stem cells in glioma and lymphoma<sup>94,126-128</sup>. In this part of our work, we provided the first evidence of an essential role for HIF1 $\alpha$  in regulating self-renewal of LSCs of CML. We demonstrated a loss of colony-forming potential and a failure to induce CML disease in the secondary recipients by HIF1 $\alpha$ <sup>-/-</sup> LSCs. Therefore HIF1 $\alpha$  signaling joins the ranks of the positive regulatory pathways in LSC maintenance, which include Wnt/ $\beta$ -catenin, hedgehog, FoxO and Bcl6 pathways.

Another exciting finding in this part of the work is that LSCs of CML are more dependent on HIF1 $\alpha$  activity than HSCs, which is based on the following evidence we observed. First, deletion of HIF1 $\alpha$  results in a more significant reduction of the self-renewal ability of LSCs, as we observed that compared to wild type LSCs, *HIF1 $\alpha$ <sup>-/-</sup>* LSCs gave rise to less colonies in all three serial replating. However, compared to WT HSCs, *HIF1 $\alpha$ <sup>-/-</sup>* HSCs generated similar numbers of colonies in the 1<sup>st</sup> and 2<sup>nd</sup> plating. Second, deficiency of *HIF1 $\alpha$*  induced apoptosis of LSCs but not HSCs. Finally, *HIF1 $\alpha$ <sup>-/-</sup>* LSCs but not HSCs displayed lower expression levels of the HIF1 $\alpha$  targets *VEGF* and *GLUT1*, and microarray analysis also showed significant changes of HIF1 $\alpha$  target genes in HIF1 $\alpha$ <sup>-/-</sup> LSCs compared to HIF1 $\alpha$ <sup>-/-</sup> HSCs. Given the observation that enhanced HIF1 $\alpha$  expression and undetectable levels of pVHL were found in cancer stem cells of

lymphoma, it is possible that leukemia stem cells require higher levels of HIF1 $\alpha$  for their maintenance than normal stem cells, thereby making them more sensitive to HIF1 $\alpha$  inhibition<sup>94,244</sup>. Together, these findings make it possible to effectively target LSCs with less harm to their normal stem cell counterparts.

However, several important questions need to be addressed in the future.

1) How does HIF1 $\alpha$  regulate the expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup>? It is known that p16<sup>Ink4a</sup> and p19<sup>Arf</sup> are encoded by the *INK4-ARF* locus, and these locus genes are epigenetically silenced in HSCs but become active upon exposure to oncogenic stress, thereby inducing cell senescence or apoptosis<sup>245</sup>. *INK4-ARF* deletions frequently occur in Ph<sup>+</sup> ALL, and *Arf* gene loss enhances oncogenicity and attenuates responsiveness to imatinib in a mouse model of BCR-ABL-induced ALL<sup>246,247</sup>. We found that HIF1 $\alpha$  deletion induced a tumor suppressor response in LSCs through induction of expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup>. We also showed that knockdown of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> rescued the defective colony-forming ability of *HIF1 $\alpha$* <sup>-/-</sup> LSCs. It is still unclear how HIF1 $\alpha$  mediates expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup>. Through analyzing the promoter sequences of p16<sup>Ink4a</sup> and p19<sup>Arf</sup>, we did not find the HIF-responsive element (HRE) that mediates the binding of HIF1 $\alpha$  to its targets, suggesting that HIF1 $\alpha$  does not directly regulate p16<sup>Ink4a</sup> and p19<sup>Arf</sup>. Therefore, there must be a mediator which links HIF1 $\alpha$  with p16<sup>Ink4a</sup>/p19<sup>Arf</sup>. It is well known that the *INK4-ARF* locus is repressed by members of the Polycomb group (PcG), including Bmi1<sup>248</sup>. *Bmi1*<sup>-/-</sup> MEFs exhibit accumulation of p15<sup>Ink4b</sup>, p16<sup>Ink4a</sup> and p19<sup>Arf</sup><sup>249</sup>. Conversely, overexpression of Bmi1 represses the expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> in HSCs, thereby rescuing the defective reconstitution ability of HSCs caused by

HIF1 $\alpha$  deficiency<sup>181</sup>. Together, these data suggest that Bmi1 might mediate the regulation of *p16<sup>Ink4a</sup>* and *p19<sup>Arf</sup>* by HIF1 $\alpha$  in LSCs. Therefore, it will be helpful to examine whether HIF1 $\alpha$  deletion down-regulates Bmi1 expression in LSCs, although a previous study showed that overexpression of HIF1 $\alpha$ , or exposure to hypoxic conditions, up-regulated Bmi1 mRNA and protein levels in tumor-initiating cells of head and neck squamous cell carcinoma<sup>250</sup>.

2) The efficiencies and effects of echinomycin or other HIF1 $\alpha$  inhibitors, such as PX-478<sup>251</sup>, on LSCs of CML need to be investigated. Although CSCs in lymphoma exhibit high sensitivity to the HIF1 $\alpha$  inhibitor, echinomycin<sup>94</sup>, it is unknown whether echinomycin has the same effect on CML LSCs. These studies will be investigated using our CML mouse model and primary CML cells from human patients.

3) What are the functions of other targets of the HIF1 $\alpha$  pathway in LSCs? Further analyzing the gene expression profiling of *HIF1 $\alpha$ <sup>-/-</sup>* LSCs will be helpful to answer this question. Additionally, we observed a lower expression level of *Alox5*, which is required for CML LSCs<sup>85</sup>, in *HIF1 $\alpha$ <sup>-/-</sup>* LSCs, suggesting that *Alox5* might be one of the downstream genes of HIF1 $\alpha$ . Therefore the relationship between *Alox5* and HIF1 $\alpha$  will need to be examined through biochemical analysis, such as the luciferase assay, ChIP, and biological assays, such as the colony forming assay.

4) It will be of interest to investigate how a hypoxic environment affects LSCs, given the critical role of HIF1 $\alpha$  in mediating the effect of hypoxia on normal hematopoiesis<sup>178,180</sup>. The interaction between HSCs and the bone marrow niche, which is considered as a relatively hypoxic microenvironment, is critical for the maintenance of

normal HSC quiescence. A recent study revealed that HSCs are hypoxic in the niche and display a higher expression of HIF1 $\alpha$ <sup>181</sup>. Accumulating evidence indicates that LSCs occupy and receive important signals from the microenvironment<sup>252</sup>. However, many fundamental questions remain to be examined. For example, do LSCs and their normal counterparts require different expression levels of HIF1 $\alpha$  for their self-renewal? What is the role of HIF2 $\alpha$  in mediating hypoxia influence on LSCs? Therefore, it is reasonable to consider hypoxia as a signal provided by the bone marrow niche for LSC maintenance, and it is necessary to investigate the molecular mechanisms of how hypoxia regulates LSCs in the future.

#### **The negative regulatory pathways----Blk and Scd1 in LSCs**

Our strategy is to identify key regulators that have essential roles in LSCs but not their normal stem cell counterparts. In this part of my work, *Blk* and *Scd1* were chosen for further functional study based on our comparison of the gene expression profiling of LSCs and HSCs. We found both *Blk* and *Scd1* are significantly down-regulated in CML LSCs, which is consistent with the results of a microarray on human CML showing that *Blk* and *Scd1* expression are markedly down-regulated in the majority of CML patients in the chronic phase, accelerated phase and blast crisis. Additionally, we identified that both *Blk* and *Scd1* function as tumor suppressors in CML through their effects on the proliferation and apoptosis of LSCs. The roles of the classic negative regulatory pathways, such as p53 and Pten, have been investigated<sup>121,253</sup>. We have shown that deletion of Pten accelerates CML development; conversely, enforced expression of Pten markedly delays the development CML<sup>121</sup>. We further proved that Pten affects the

reconstitution ability of CML LSCs via regulating the cell cycle and apoptosis of LSCs.

In this part of the thesis, our findings reveal that *Blk* and *Scd1* represent two novel negative regulators for CML LSCs, thereby contributing to understanding the genetic basis and underlying biology of CML LSCs.

Most importantly, in this part of my work, we found that both *Blk* and *Scd1* specifically affect CML LSCs but do not markedly affect normal hematopoiesis. Compared to wild type mice, *Blk*<sup>-/-</sup> or *Scd1*<sup>-/-</sup> mice display similar percentages of different stem cell populations including long term and short term HSCs. A series of functional assays, such as a competitive repopulation assay and rescue assay were performed to assess the functions of *Blk*<sup>-/-</sup> or *Scd1*<sup>-/-</sup> HSCs in normal hematopoiesis. We found that deletion of *Blk* or *Scd1* did not obviously affect the function of normal HSCs. Given their critical role in CML LSCs, thereby affecting the development of CML, we are confident to propose that *Blk* and *Scd1* are unique regulators of CML LSCs but not their normal stem cell counterparts. Interestingly, using high-throughput screening, Weinberg and colleagues identified specific inhibitors of epithelial CSCs<sup>131</sup>, implying the existence of molecular pathways specific to CSCs that could serve as effective and selective therapeutic targets. In our lab, we identified the *Alox5* gene as an important regulatory gene in LSCs but not normal HSCs<sup>85</sup> and showed that *Alox5* deficiency impaired CML development by blocking stem cell differentiation. Therefore, the findings in this part of my work strengthen the feasibility of specifically targeting LSCs in the treatment of CML.

The major issue in CML biology is to understand why LSCs are insensitive to TKI monotherapy. Another exciting finding in this thesis might provide the molecular mechanism for the insensitivity of LSCs to TKI. We found that the down-regulation of *Blk* is independent of BCR-ABL kinase activity, shown by the finding that imatinib treatment failed to restore the down-regulation of *Blk* in LSCs. On the one hand, this finding in here is in line with our previous data showing that Src family kinases are independent of BCR-ABL kinase activity, as inhibition of BCR-ABL kinase activity cannot affect the activation of Src kinases by BCR-ABL<sup>86</sup>. This finding confirms the idea we proposed previously that there is BCR-ABL kinase activity-independent pathways in LSCs<sup>254</sup>. On the other hand, considering the recent studies demonstrating that human CML stem cells do not depend on BCR-ABL activity for their survival<sup>64,84</sup>, the study in this part suggests that the BCR-ABL kinase independent pathways are common phenomena in CML stem cells, and provides the explanation of how TKIs cannot eliminate CML LSCs. This suggests that TKI therapy alone is not enough for curing CML. In our lab, we identified several genes, such as *Alox5* and *Tph1*, which are independent of BCR-ABL kinase activity<sup>85</sup>. Therefore, from a therapeutic standpoint, the work described in this part of the thesis provides the rationale for the alternative strategies that are developed to target these kinase independent pathways for eliminating CML LSCs.

However, several important questions remain to be answered.

1) What is the underlying mechanism by which *Blk* suppressed LSCs? Although we have shown that p27 mediates the inhibitory effect of *Blk* on LSCs, it is likely that

there are other downstream Blk target genes. For example, we observed that overexpression of Blk markedly increased the apoptosis of LSCs, implying that Blk might regulate the apoptotic pathways. Therefore, it is important to investigate whether the apoptotic related genes (such as *p53* and *Bcl2*) mediate the function of Blk in LSCs.

2) Regarding Scd1, it remains unclear how BCR-ABL inhibits Scd1 expression. As it is known that Scd1 expression is regulated by several transcriptional factor, including SREBP-1c, PPAR, and LXR, it is important to investigate whether these factors mediate the downregulation of Scd1 in LSCs.

3) The detailed mechanisms of how Scd1 suppresses CML development via affecting the function of LSCs need to be investigated further. How does Scd1 influence the expression of Pten, p53, and Bcl2? What is the mediator between Scd1 and Pten, p53 or Bcl2? Do other pathways, excluding Pten, p53 and Bcl2, mediate the effect of Scd1 on LSCs? For example, a previous study showed that deletion of Scd1 activates the insulin signaling pathway<sup>173</sup>. On the other hand, BCR-ABL activates IGF-1 signaling, and blocking this pathway with small molecule drugs or shRNA decreases proliferation and enhances apoptosis of leukemia cells<sup>255</sup>. Therefore, it is reasonable to assess whether the insulin pathway is responsible for the effect of Scd1 on LSCs.

4) The mechanism by which a PPAR $\gamma$  agonist inhibits LSCs is unclear. PPARs are ligand-binding transcription factors belonging to the nuclear receptor family. PPARs are involved in regulating lipid metabolism, cell growth and survival. It has been shown that activation of the PPAR pathway with a dual  $\alpha$  and  $\gamma$  ligand TZD18 induces apoptosis and inhibits proliferation of human CML cells<sup>256,257</sup>. Although rosiglitazone synergized

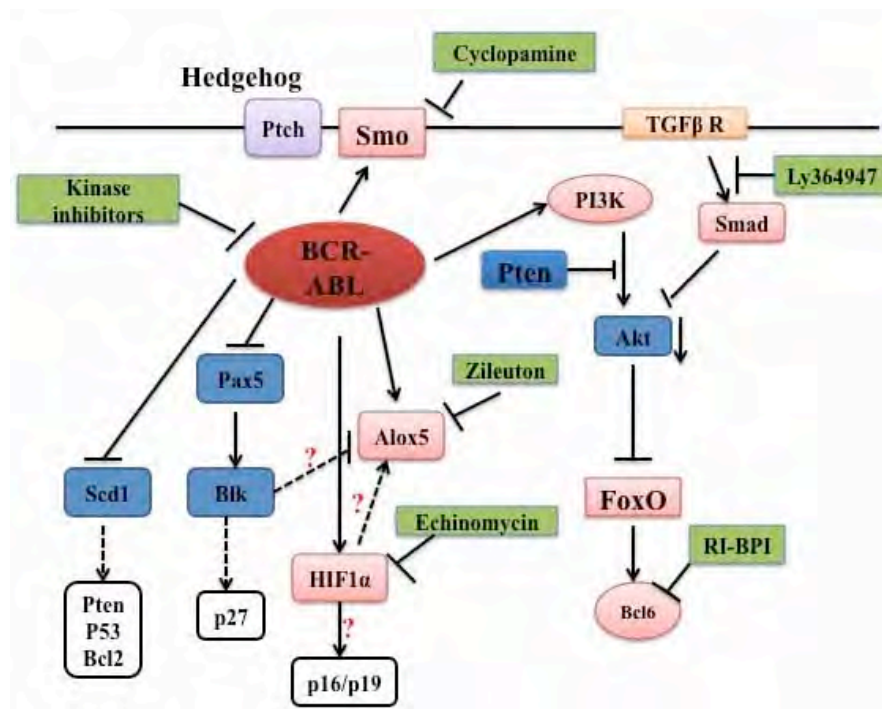
with imatinib to delay CML development, rosiglitazone alone failed to do so. One possibility is that rosiglitazone alone is not effective enough to prevent lung hemorrhages, a major cause of death of CML mice. Another possibility is that the level of Scd1 induced by rosiglitazone is not high enough to delay CML development. PPAR agonists are anti-inflammatory agents as they inhibit expression of several proinflammatory cytokines and most chemokines<sup>258</sup>. The role of anti-inflammatory agents on LSCs is supported by our previous study showing that the anti-inflammatory drug Zileuton that inhibits the function of Alox5 gene and prolongs survival of CML mice<sup>85</sup>. Therefore, it is reasonable to hypothesize that the Alox5 inhibitor may synergize with PPAR agonists in the elimination of LSC. The *in vitro/in vivo* treatment assay can be used to observe if a deeper response can be predicted.

### **Strategy for targeting LSCs---- combination therapy**

Targeting leukemia stem cells has become a common proposal for curing CML. Taken together, based on the findings in this thesis, I propose that combination therapy of TKI with drugs that target the key regulators of LSCs will be the promising and effectively therapeutic strategy for CML treatment (Figure 1). Several lines of evidence indicate that inactivation of key pathways for CML stem cell maintenance could contribute to CML therapy. Pharmacological blockade of Hedgehog signaling by cyclopamine<sup>92,110</sup>, inhibition of the TGF $\beta$ -FoxO pathway by Ly364947, inactivation of Bcl6 by the retro-inverso Bcl6 peptide inhibitor RI-BPI, or inhibition of Alox5 by zileuton, impair CML development by suppressing LSCs. Similarly, inhibition of the HIF1 $\alpha$  pathway by echinomycin would effectively eliminate LSCs. Interestingly, given



the specific roles of *Blk* and *Scd1* in CML LSCs, either restoration of *Blk* expression, or enhancement of *Scd1* activity, provides an attractive strategy for specifically targeting LSCs in CML.



**Figure 1. The combination of inhibiting BCR-ABL by TKIs and targeting key regulators of LSCs in CML treatment.** TKI treatment plus inhibition of positive pathways, or restoration the expression of negative regulators could effectively target LSCs for curing CML.

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